THE UNIVERSITY
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Cellular regulation of cortisol *in vivo* by 11-beta hydroxysteroid dehydrogenase type 1

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Declaration

I hereby declare that this thesis was written by me. The data published in this thesis are the result of my own work with the exception of the acknowledged contributory work by Dr G Jones in Chapter 3. The work in this thesis has not previously been submitted for any other degree or qualification.

Anna Anderson

Edinburgh, March 2016

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Abstract

Glucocorticoid excess as a result of Cushing's syndrome or pharmacological treatment can result in the development of obesity and type 2 diabetes mellitus (T2DM). The reactivation of cortisone to cortisol is catalysed by 11βHSD1 which is expressed widely but notably in adipose tissue and liver. Studies have shown dysregulation of cortisol in these tissues with obesity potentially promoting the development of T2DM. Inhibition of 11βHSD1 has been attempted as a novel treatment for T2DM with observed improvement in glycaemic control, body weight and blood pressure. The efficacy of such agents has been disappointing with few reaching phase 2 trials. With recent evidence of bidirectional activity of 11βHSD1 *in vivo* it becomes apparent that dysregulation may occur at an intracellular rather than tissue level.

In this thesis I address several key outstanding questions concerning the physiology and regulation of 11βHSD1 including:

1. Whether combined therapy with metformin alters 11βHSD1 activity and obscures the efficacy of 11βHSD1inhibitors;
2. Whether the contribution of 11βHSD1 to local cortisol concentrations has been under-estimated by considering total rather than free cortisol turnover; and

3. Whether recycling between cortisol and cortisone in adipose tissue and skeletal muscle in obesity is a neglected feature of 11βHSD1 biochemistry and function.

Eight obese healthy men with and without type 2 diabetes were recruited to a randomised placebo controlled cross over trial. They received 4 weeks treatment with metformin and placebo. Participants with T2DM additionally received gliclazide as a further control. Using the deuterated tracer D4-cortisol 11βHSD1 activity was measured. Metformin treatment increased whole body 11βHSD1 in both groups postulated as a result of improved insulin sensitivity.

11βHSD1 is located within cells and so contributes to free tissue cortisol concentrations but perhaps less so to total (protein-bound) cortisol in plasma. It has been shown that 11βHSD1 contributes almost half of total circulating cortisol concentrations at rest. This measurement relied upon blood sampling during steady state deuterated cortisol (D4-cortisol) infusion with measurements of total (free plus protein bound) cortisol which may have underestimated true 11βHSD1 activity. This was therefore investigated by comparing 11βHSD1 activity as calculated using total compared with free cortisol tracer enrichments. Equilibrium dialysis was performed separating free from bound portions in plasma samples taken from healthy volunteers who received D4-cortisol infusion. Analysis revealed similar measurements of 11βHSD1 activity using free compared with total cortisol implicating rapid turnover of glucocorticoids between the free and bound pools.

On first discovery 11βHSD1 was seen to be a dehydrogenase enzyme *in vitro*. Later work recognised reductase activity *in vivo* and up until recently 11βHSD1 has been

viewed as a predominantly reductase enzyme. As with other enzymes in the same family, the ability to catalyse both reductase and dehydrogenase depends upon the availability of substrate and co substrate. Whether dysregulation of 11βHSD1 in the settings of obesity and T2DM is the result of alteration in directionality at a cellular level is not known. Firstly bidirectional activity of 11βHSD1 was confirmed *in vitro* using HEK-293 cells stably transfected with 11βHSD1. The influence of obesity and acute perturbation with hyperinsulinaemia was subsequently investigated *in vivo* in a random order cross over single blinded case control study involving ten normal weight and ten obese healthy male volunteers. D4-cortisol and deuterated cortisone (D2-cortisone) were infused for the measurement of reductase and dehydrogenase activity of 11βHSD1 respectively with measurements taken across forearm muscle and abdominal subcutaneous adipose tissue. Across whole body, lean and obese individuals displayed similar 11β-reductase and 11β-dehydrogenase activity. Across tissue, 11β-reductase and 11β-dehydrogenase activity was different from zero across adipose tissue in obese individuals and across skeletal muscle in lean individuals providing further evidence of tissue specific differences in 11βHSD1 with obesity. With the addition of hyperinsulinaemia, reductase and dehydrogenase activity was somewhat increased in lean individuals although there was no statistically significant difference between lean and obese individuals. Across tissue there was a trend for obese individuals to display increased 11β-reductase activity across adipose tissue with hyperinsulinaemia. Comparing the rates of reductase and dehydrogenase activity revealed predominantly reductase activity across tissue in obese and dehydrogenase activity in lean individuals. The development of direction specific

inhibitors targeting reductase activity by 11βHSD1 may prove efficacious for the treatment of obesity.

In conclusion, 11βHSD1 acts as a bidirectional enzyme *in vitro* and *in vivo*. Overall directionality of enzyme activity is altered in a tissue specific manner in the setting of obesity. We have shown that this intracellular regulation of cortisol is reflected equally in the metabolically active free pool and total plasma pool. The efficacy of 11βHSD1 inhibitors as novel agents for the treatment of T2DM and coexisting obesity is not diminished by co-prescription with metformin but may prove more efficacious through the development of reductase specific inhibitors.

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Abbreviations

Abbreviation	
11βHSD	11 β-hydroxysteroid dehydrogenase enzymes
11βHSD1	11 β-hydroxysteroid dehydrogenase type 1
11βHSD2	11 β-hydroxysteroid dehydrogenase type 2
AVP	Arginine vasopressin
Allo-THF	Allo-tetrahydrocortisol
BMI	Body mass index
BP	Blood pressure
CBG	Corticosteroid binding globulin
CRH	Corticotropin-releasing hormone
CRHR1	Corticotropin releasing hormone receptor 1
CRHR2	Corticotropin releasing hormone receptor 2
GABA-BDZ	Gamma-aminobutyric acid-benzodiazepine
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate

FAI	Free androgen index
GR	Glucococorticoid receptor
GRE	Glucocorticoid response element
GWAS	Genome wide association study
HbA1c	Glycated haemoglobin
H6PDH	Hexose-6-phosphate dehydrogenase
HEK293-11βHSD1	Human Embryonic Kidney cells stably transfected with 11βHSD1
HPA axis	Hypothalamic-pituitary-adrenal axis
LDL	Low density lipoprotein
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
MR	Mineralocorticoid receptor
NAD	Nicotinamide adenine dinucleotide

NADP/ NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
POMC	Pro-hormone pro-opiomelanocortin
Ra	Rate of appearance
RCL	Reactive centre loop
RNA	Ribonucleic acid
SD	Standard deviation
SDR	Short-chain dehydrogebase/reductase enzyme
SNP	Single nucleotide polymorphism
T2DM	Type 2 diabetes mellitus
THE	Tetrahydrocortisone
THF	Tetrahydrocortisol

Publications

Anderson A, Walker B. **11β-HSD1 inhibitors for the treatment of type 2 diabetes mellitus and cardiovascular disease.** Drugs: 2013; 13: 1385 – 93

Bolton JL, Hayward C, Direk N, Lewis JG, Hammond GL, Hill LA, Anderson A, Huffman J, Campbell H, Rudan I, Wright A, Hastie N, Wild SH, Velders FP, Hoffman A, Uitterlinden AG, Lahti J, Räikkönen K, Kajantie E, Widen E, Palotie A, Eriksson JG, Kaakinen M, Järvelin MR, Timpson NJ, Davey Smith G, Ring SM, Evans DM, St Pourcain B, Tanaka T, Milaneschi Y, Bandinelli S. Ferrucci L, Van der Harst P, Rosmalen JG, Bakker SJ, Verweij N, Dullaart RP, Mahajan A, Lindgren CM, Morris A, Lind L, Ingelsson E, Anderson LN, Pennell CE, Lye SJ, Mathews SG, Eriksson J, Mellstrom D, Ohlsson C, Price JF, Strachan MW, Reynolds RM, Tiemeier H, Walker BW, COrtisol NETwork (CORNET) corsortium. **Genome wide association identifies Common variants at the SERPINA6/SERPINA1 locus influencing plasma cortisol and corticosteroid binding globulin.** PLOS Genetics 2014; 10; 10: e1004474

Anderson A, Andrew R, Homer NZ, Jones GC, Smith K, Livingstone DE, Walker BW, Stimson RH. **Metformin increases cortisol regeneration by 11βHSD1 in obese men with and without type 2 diabetes mellitus.** J Clin Endocrinol Metab. 2016; 101: 3787 – 3793

Presentations

Metformin increases in vivo 11β-HSD1 activity in obese men with and without type 2 diabetes mellitus.

- Oral and poster presentation at 15th Annual Rachmiel Levine Diabetes and Obesity Symposium, San Diego, March 2015
- Poster presentation and poster preview presentation at ENDO conference, San Diego, March 2015
- Oral presentation at Caledonian Society for Endocrinology and Diabetes annual meeting, Dunkeld, November 2015

Rapid equilibriation of cortisol between the free and total plasma pools

- Poster presentation at British Endocrinology Society meeting, November 2015

Awards

The Rachmiel Levine Scientific Achievement Award (March 2015) for abstract entitled

Metformin increases in vivo 11β-HSD1 activity in obese men with and without type 2 diabetes mellitus.

Abstract presentation prize at Caledonian society for Endocrinology and Diabetes meeting (November 2016)

Tissue specific regulation of recycling between cortisol and cortisone by insulin and obesity

Chapter 1: Introduction

1.1 Cortisol function

Glucocorticoids regulate metabolic, cardiovascular and immune parameters allowing adaptive changes to be made in response to an altering environment with the aim of maintaining whole body homeostasis. Such adjustments include mobilisation or storage of energy and modulation of immune response and cardiovascular parameters. Acute elevation in circulating cortisol occurs physiologically (such as morning wakening and eating) and during pathological stress (such as infection) and psychological stress. Chronic stimulation of this mechanism can be detrimental, coined allostatic load (McEwen 1998). The detrimental effects of excess circulating cortisol are easily seen with exogenous glucocorticoid administration (Wei, et al. 2004) and autonomous cortisol generation in spontaneous Cushing's syndrome (Giordano, et al. 2011). Such individuals develop central adiposity, hypertension dyslipidaemia and altered glucose regulation. Such features are additionally encountered in the increasingly prevalent metabolic syndrome. Contrary to expectation cortisol concentrations have consistently been found to be lower in obesity (Rask, et al. 2001; Rask, et al. 2002). Excretion rates of cortisol and its metabolites in urine are concomitantly elevated (Marin, et al. 1992; Strain, et al. 1982; Strain, et al. 1980) implicating dysregulation of cortisol with obesity in a tissue specific manner (Rask et al. 2001; Rask et al. 2002).

Clear evidence exists of a relationship between elevated circulating cortisol and the development of cardiovascular disease (Etxabe and Vazquez 1994; Wei et al. 2004). More subtle changes in circulating cortisol, such as higher morning cortisol concentrations, have been associated with cardiovascular risk factors including hypertension (Bjorntorp, et al. 1999; Litchfield, et al. 1998; Whitworth, et al. 1995),

dyslipidaemia (Walker, et al. 2000) and altered glucose regulation (Bjorntorp et al. 1999). An association of such diurnal variations in cortisol concentrations with hard endpoint cardiovascular disease is harder to elicit (Alevizaki, et al. 2007; Reynolds, et al. 2009).

This thesis concentrates on the role of cortisol in regulating metabolic activity in adipose tissue, skeletal muscle and liver, and the modulation of cortisol action by local activity of the enzyme 11 β-hydroxysteroid dehydrogenase type 1.

1.2 Cortisol structure

Glucocorticoids are a sub-family of steroid hormones; all steroid hormones have the same basic structure consisting of three cyclohexane rings and one cyclopentane ring. The addition of side chain moieties around this structure results in the hormone specific function. Each ring is labelled A to D with sequential numbering of the carbon atoms around each ring in turn allowing identification and standardised naming of each compound (Figure 1.1). Cortisol is defined structurally by its hydroxyl group at position 11. This contrasts with the inactive form of the glucocorticoid, cortisone which differs only by the loss of two hydrogen atoms generating a carbonyl functional group allowing easy conversion between the two. Dehydrogenation of cortisol generating cortisone occurs by the action of the 11β-hydroxysteroid dehydrogenase enzymes, namely type 1 (11βHSD1) and type 2 (11βHSD2). The function of 11βHSD1 will be discussed in detail in the relevant sections below.

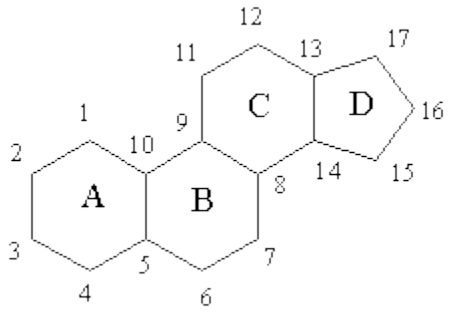


Figure 1.1 Generic structure of steroid compounds

1.3 Cortisol production

1.3.1 Central regulation

Cortisol is synthesised via a multi-step enzymatic reaction in the zona fasciculata, the middle layer, of the adrenal gland. This process is initiated and controlled through the hypothalamic-pituitary-adrenal axis (HPA axis). Through a negative feedback loop fine control of circulating glucocorticoids can be achieved.

The hypothalamus receives afferent signals from neuroendocrine pathways, inflammatory mediators and from the limbic system controlling the secretion of corticotropin-releasing hormone (CRH). Potentiating the effect of CRH is vasopressin (AVP) which is co-secreted by the parvocellular neurons of the hypothalamic paraventricular nucleus. Inflammatory cytokines, serotonin and acetylcholine have a positive stimulating effect on the hypothalamus whereas substance P, gamma-aminobutyric acid-benzodiazepine (GABA-BDZ) and opioid peptides have an inhibitory effect on CRH release.

Neuronal stimulation of the hypothalamus releases CRH into the portal vessel system activating the synthesis of the pro-hormone pro-opiomelanocortin (POMC) by the

anterior pituitary. This is cleaved producing several peptides including adrenocorticotropic hormone (ACTH). Through G-protein coupled receptor, ACTH initiates steroidogenesis within the adrenal cortex.

A rapid 'fight and flight' response to stress is stimulated by CRH and AVP through corticotropin releasing hormone receptor 1 (CRHR1) initiating behavioural, neuroendocrine and autonomic response to stress.

In the short term the activation of the HPA axis to psychological or environmental stress aims to restore homeostasis. Regulation of whole body cortisol concentrations by this feedback mechanism has been seen to be disrupted with obesity (Mattsson, et al. 2009) and glucose intolerance (Reynolds, et al. 2001b) and may potentiate the development of cardiovascular disease (Reynolds et al. 2001b).

Per Bjorntorp first identified the potential association between altered cortisol regulation through the HPA axis and the development of features of the metabolic syndrome and with it an increased risk of cardiovascular disease (Björntorp 1988). This was later corroborated in men of low birth weight who were observed to have altered HPA axis function and increased development of the metabolic syndrome (Reynolds, et al. 2001a). Obese individuals have been observed to have heightened response of pituitary control both to stimulation, by CRH (Rask et al. 2002), and suppression, by dexamethasone (Rask et al. 2001). A study by Mattsson et al. revealed both the synergistic effect of glucocorticoid and mineralcorticoid receptor activation on HPA axis negative feedback as well as reduced responsiveness of this feedback in obese individuals (Mattsson et al. 2009).

This evidence provides the basis for the development of 11βHSD1 inhibitors for the treatment of components of the metabolic syndrome and as such with potential to reduce the risk of future development of cardiovascular disease.

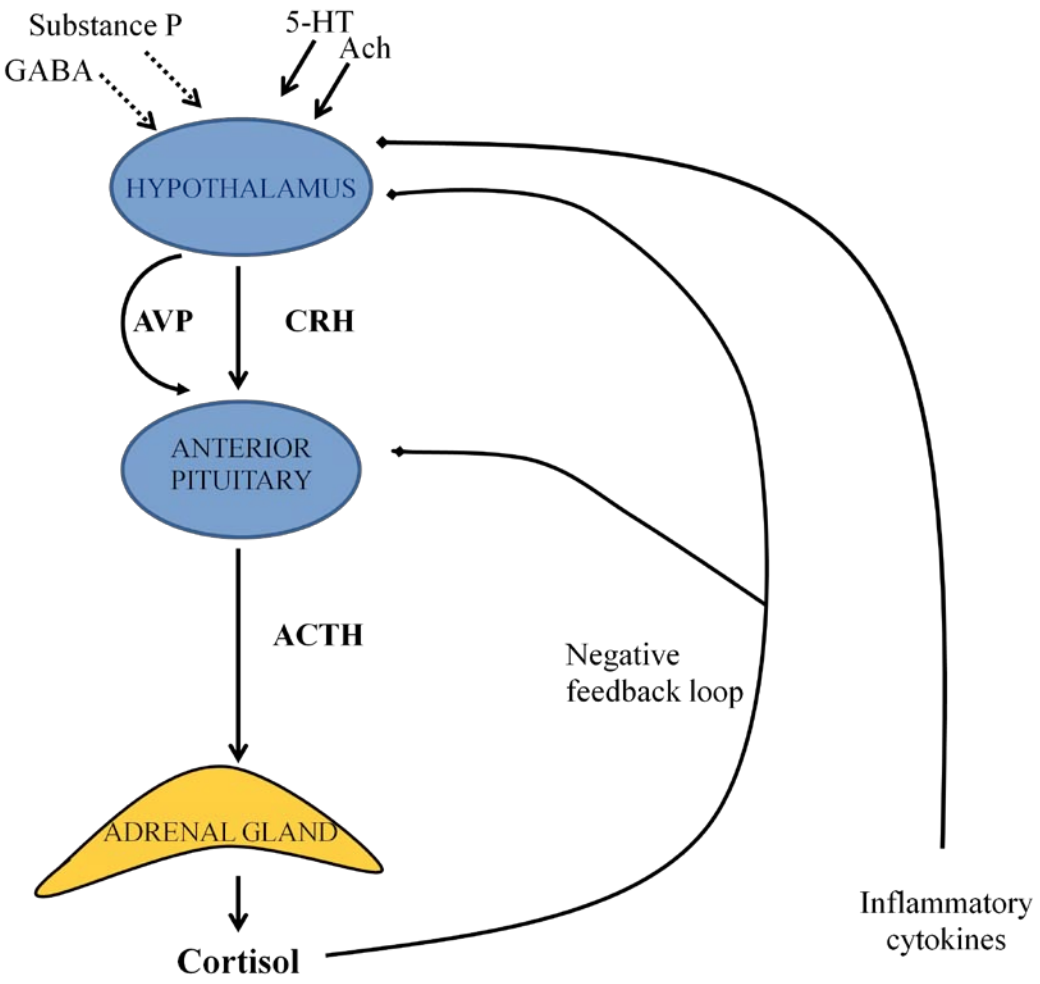


Figure 1.2 Central regulation of cortisol production through the hypothalamic-pituitary adrenal axis

5-HT; serotonin: Ach; acetylcholine: GABA;gamm-aminobutyric acid :AVP; vasopressin: CRH; corticotropin-releasing hormone

1.3.2 Adrenal steroidogenesis

ACTH initiates steroidogensis by binding to melanocortin-2 receptor (MC2R) in the adrenal cortex. The initial and rate limiting step in steroid production is the incorporation of the starting material, cholesterol, into the mitochondria. This is hydrolysed by CYP11A1 generating pregnenolone. The hydroxyl group at carbon position 3 is subsequently oxidised by 3β-hydroxysteroid dehydroengase type 2 forming progesterone. Pregnenolone and progesterone can follow one of three pathways, depending on the location within the adrenal gland and as such the availability of specific enzymes resulting in the formation of (a) the mineralococorticoid aldosterone, primarily from the outer zona glomerulosa, (b) cortisol from the middle zona fasciculate and (c) androgens from the innermost zona reticularis. The production of cortisol necessitates the presence of 17α-hydroxylase for the generation of 17 hydroxy-pregenolone or 17 hydroxy-progesterone from pregenelone and progesterone respectively. From here enzymatic reactions involve 21 hydroxylase and 11β-hydroxylase resulting in the end product cortisol. Failure of one of these pathways, through enzyme deficiency or malfunction, can result in an accumulation of ‘upstream’ intermediate compounds resulting in divergence of the pathway in favour of one or other of the end products.

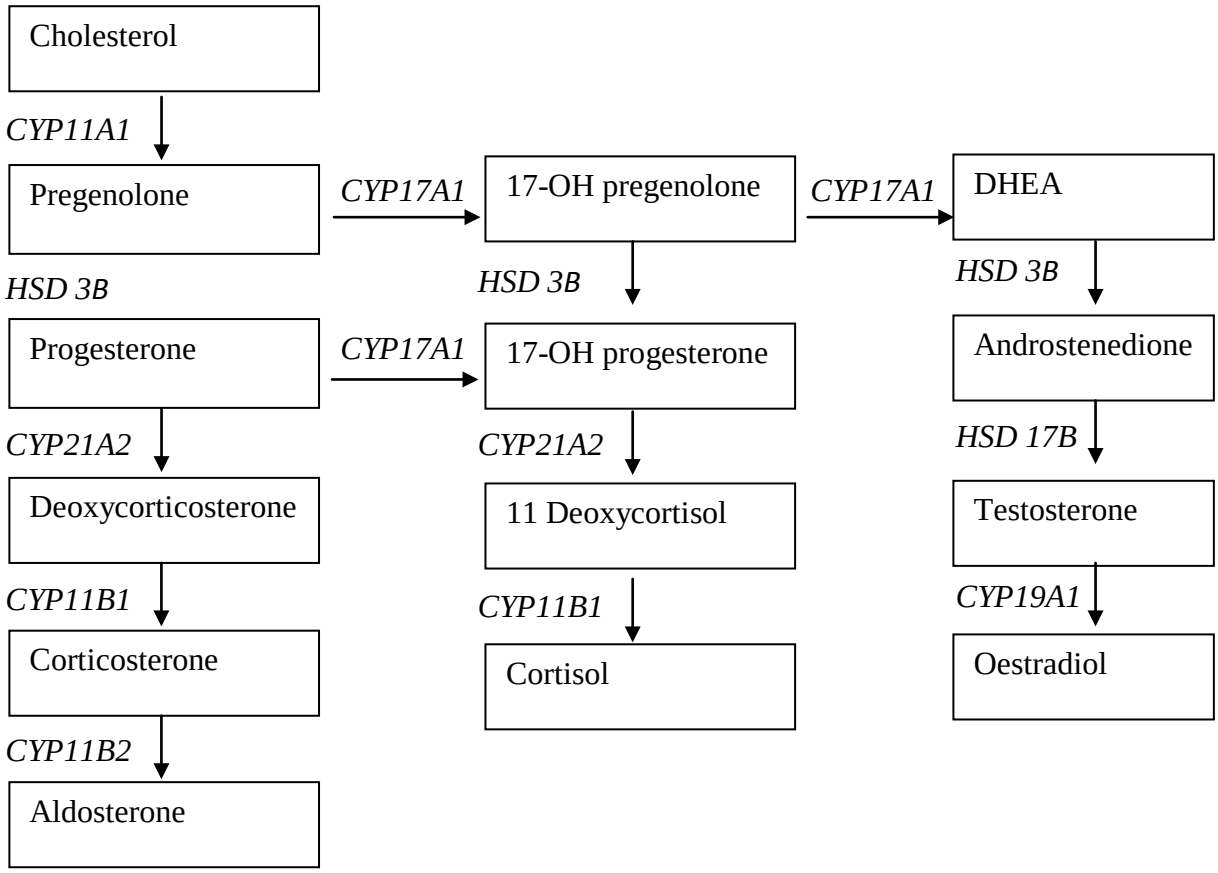


Figure 1.3 Adrenal steroidogenesis pathway

As cortisol is secreted on demand, acute adjustments can be made to bio-available steroid. Both the hypothalamus and pituitary glands detect circulating cortisol levels with inhibition of CRH and ACTH production when adequate levels are sensed in a classical negative feedback loop.

1.3.3 Cortisol secretion

Cortisol secretion follows a natural circadian rhythm with elevated concentrations upon wakening reducing to a nocturnal nadir. Smaller fluctuations additionally occur throughout the day in an ultradian rhythm in relation to eating and in response to stress and alterations in the external environment. Such sensitive manipulations are feasible through adjustments in the pulsatile nature in which ACTH is secreted. Elevated morning concentrations is primarily as a result of increased amplitude (Veldhuis, et al. 1990) whereas nocturnal nadir is through a reduction in the frequency of these pulses (Horrocks, et al. 1990).

1.4 Corticosteroid Binding Globulin

Over 90% of cortisol circulates bound to the carrier protein corticosteroid binding globulin (CBG). A small proportion is additionally bound to albumin leaving less than 5% to circulate free. It is this unbound portion which diffuses into the extravascular compartment and is metabolically active. Binding and dissociation of cortisol to CBG is a dynamic process and can therefore be seen as an additional level of regulation in the control of cellular cortisol action. Binding affinity of cortisol to CBG is altered with fluctuations in temperature (Cameron, et al. 2010) and pH (Mickelson, et al. 1981) with high temperatures and acidic conditions instigating cortisol dissociation. Cortisone competes with cortisol for the binding site on CBG but with an affinity 10 times less than cortisol (Dunn, et al. 1981).

1.4.1 CBG as a carrier protein

CBG is a member of the serine protease inhibitors (serpin) family although it lacks any inhibitory action on protease enzymes. Like other members of the serpin family

it undergoes dramatic conformational change from stressed to relaxed state. In the stressed state the cortisol binding pocket, or reactive centre loop (RCL), is exposed whereas in the relaxed state it is concealed by the main β sheet of the protein (Figure 1.4). This conformational change, catalysed by cleavage of the RCL by neutrophil elastase, results in around a nine fold reduction in binding affinity (Chan, et al. 2013; Hammond, et al. 1990). It is hypothesised that this allows greater dissociation of cortisol at sites of inflammation although studies have only proven this *in vitro (Hammond et al. 1990)*. Antibodies directed at the exposed reactive centre loop in stressed CBG molecules have been developed. Paired measurement of total CBG concentrations then allows quantification of conformational change in a variety of situations (Lewis and Elder 2011). Through their use one study has shown a direct relationship between sepsis of increasing severity and circulating levels of cleaved low affinity CBG (Nenke, et al. 2015). Shock secondary to sepsis resulted in greater cleavage of CBG compared to shock of other cause (such as cardiogenic) despite non-significantly different total white cell count implicating differential white cell count, for example neutrophils, in mediating this conformational change.

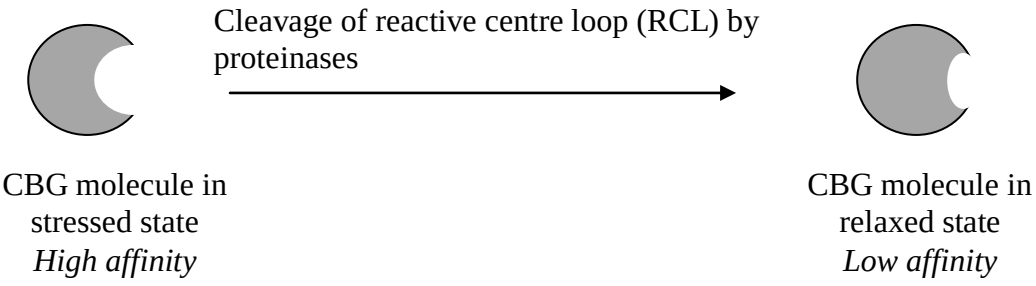


Figure 1.4 Schematic of cleavage of corticosteroid binding globulin (CBG) from stressed to relaxed state

Unlike albumin, CBG has a high affinity for cortisol but low binding capacity with saturation occurring at cortisol concentrations of around 500 nM. With cortisol circulating at around this concentration throughout most of the circadian curve, an exponential rise in free cortisol is precipitated with any additional cortisol production.

1.4.2 Alteration in CBG binding affinity and capacity

CBG binding capacity is influenced by other circulating hormone levels including insulin, oestrogens and glucocorticoids. Stimulated insulin secretion through the use of an intravenous glucose infusion results in an acute reduction in CBG concentrations postulated to be the result of reduced hepatic synthesis (Fernandez-Real, et al. 2001). This effect is lost in the presence of insulin resistance. Oestrogens increase CBG production (Musa, et al. 1965) whilst glucocorticoids have a negative feedback role (Schlechte and Hamilton 1987).

Akin to other circulating peptides, CBG is open to glycosylation. Six consensus sites are available for modification. Glycosylation at amino acid Asn^{238} is necessary for the correct folding and generation of the steroid-binding site during biosynthesis (Avvakumov and Hammond 1994). Post-transcription glycosylation plays a significant role in altering binding affinity with glycosylated CBG demonstrating a nine fold greater affinity for cortisol (Chan et al. 2013). Whether higher circulating glucose concentrations such as with diabetes mellitus results in such alterations remains unknown.

1.4.3 CBG transcription and mutations

CBG is encoded from a 19kb gene (SERPINA6) on Chromosome 14. This sits in close proximity to the gene encoding α-1 antitrypsin (SERPINA1). Alpha-1 antitrypsin is a further member of the serpin protease family which inhibits the action of neutrophil elastase. The effects of inherited deficiency of α-1 antitrypsin on respiratory and gastrointestinal function are well understood but potential effects on CBG binding affinity and circulating cortisol concentrations is unknown.

The first kindred of CBG mutation was identified as a result with a lack of response of CBG to increase sufficiently with oestrogen treatment (Doe, et al. 1965). Since then a variety of CBG mutations have been identified. The Leuven (Van Baelen, et al. 1982) and Lyon (Emptoz-Bonneton, et al. 2000) mutations result from a single amino acid substitution resulting in altered coupling mechanism between steroid and binding site and a resultant reduction in cortisol binding affinity (Klieber, et al. 2007). Individuals holding these mutations display symptoms of asthenia, depression and occasionally hypertension thought to be the result of relative glucocorticoid insufficiency.

Following presentation with symptoms consistent with hypoadrenalism, inadequate response to synthetic ACTH yet normal urinary free cortisol concentrations, the null mutation with complete loss of CBG was discovered (Torpy, et al. 2001). This results from a base substitution and subsequent premature stop codon preventing CBG transcription. Unexplained fatigue and hypotension have variably been reported in such individuals. A partial loss of CBG transcription to around 50% has additionally been observed in a young boy presenting with fatigue and headache (Torpy, et al. 2012). It must be clarified that the aforementioned mutations are all

rare. A much larger variety of single nucleotide polymorphisms (SNP) do, however, exist with variable consequences on production and/or function of CBG (Simard, et al. 2015). Symptomatology from these mutations is diverse potentially leading to under-diagnosis and the generation of skepticism as to extent of their impact. Compounding factors such as the environmental have been suggested to play a role (Cizza, et al. 2011). It is debated whether individuals with chronic fatigue syndrome have relative glucocorticoid insufficiency and certainly there is evidence of impaired HPA axis function in some individuals (Demitrack, et al. 1991). With a preponderance for a particular SNP, CBG function may play a role in its development although none of the well recognized mutations outlined above have been proven in such cases (Gagliardi, et al. 2010).

Genetic polymorphisms of CBG have been shown to explain a proportion (<1%) of the variance in circulating cortisol concentrations between individuals. A recent genome wide association meta-analysis (GWAS) explored the effect of single nucleotide polymorphisms (SNPs) on morning cortisol concentrations. Genetic polymorphisms at a locus on Chromosome 14 spanning both SERPINA6 and SERPINA1 correlated significantly ($P<5x10^{-8}$) with morning cortisol concentrations and were observed to influence total binding capacity of CBG as well as influencing CBG cleavage (Bolton, et al. 2014).

1.4.4 CBG and the metabolic syndrome

CBG concentrations have been observed in a large population study to be inversely related to indices of obesity including BMI, waist to hip ratio as well as with blood

pressure (Fernandez-Real, et al. 2002). As a state of chronic inflammation this may be the result of down regulated synthesis. With recognised HPA axis dysfunction in association with obesity (Mattsson et al. 2009), lower CBG levels may help maintain adequate cellular glucocorticoid exposure. Certainly, evidence exists for enhanced adipose tissue proliferation and differentiation in the presence of lower circulating CBG concentrations which is not explained by either GR or 11βHSD1 action (Joyner, et al. 2003). This may contribute to an increased prevalence of obesity with relative or complete CBG deficiency (Roitman, et al. 1984).

Association between CBG and other markers of the metabolic syndrome such as insulin resistance are less consistent (Fernandez-Real, et al. 1999; Fernández-Real, et al. 2000; Lewis, et al. 2004).

1.5 Glucocorticoid receptor and signalling pathway

Intracellular activation of the glucocorticoid receptor (GR) by glucocorticoids results in the initiation of a wide variety of processes aimed at maintaining homeostasis. Alternative splicing in the generation of GR provides functionally distinct subtypes allowing tissue and regulatory specificity (Lewis-Tuffin and Cidlowski 2006). Glucocorticoids bind to the ligand binding domain, a hydrophobic pocket embedded within the protein. This dimerised product then translocates to the nucleus where it binds with high affinity to target DNA sequences known as Glucocorticoid Response Element (GRE). GREs can either positively enhance or down regulate gene transcription. The GRE sequence itself influences gene transcription rates through the recruitment of coregulators and chromatin remodelling complexes (Rosenfeld

and Glass 2001). Proinflammatory transcription factors such as activator protein-1 and NF-κB tether to GR or co-localise to GRE down regulating transcriptional activity. This is one means by which glucocorticoids exert their anti-inflammatory and immunosuppressive effect (Newton and Holden 2007). Upregulation of gene transcription is promoted by proteins such as STAT (signal transducer and activator of transcription) molecules which act synergistically with GR (Rogatsky and Ivashkiv 2006).

Glucocorticoid receptors are open to post translational modification through the action of mitogen activated protein kinase (MAPK), cyclin dependent kinases and glycogen synthase kinase-3 (Oakley and Cidlowski 2011). The best studied modification is phosphorylation (Beck, et al. 2009) which not only modifies gene transcription (Miller, et al. 2005) but may influence the half life and stability of the protein (Webster, et al. 1997). The MAPK signalling pathway regulates the generation of pro-inflammatory proteins. By inhibiting this pathway, glucocorticoids regulate cellular inflammation. In turn MAPK has the ability to negatively feedback through post translational modification of GR (Ayroldi, et al. 2012).

In contrast to transcriptional effects of GR which take a few hours to initiate, non genomic effects such as blockage of neuronal signals through high-voltage-gated-calcium channels and inhibition of inflammatory cascade through inhibition of arachidonic release occurs within minutes (Song and Buttgereit 2006; Tasker, et al. 2006).

As ligands, glucocorticoids are not exclusive to GR but bind to mineralocorticoid receptors (MR) with equivalent, if not higher binding affinity than the

mineralcorticoiod, aldosterone (Funder 2005). The lower abundance of MR compared with GR and the role of glucocorticoid inactivating enzymes such as 11βHSD2 help prevent inappropriate activation of MR by cortisol.

Glucocorticoid receptors are widely distributed and diverse with as much as 20% of the human genome being regulated by glucocorticoids (Galon, et al. 2002). Interaction of GR with GRE occurs in pulses rapidly cycling on and off every few seconds allowing optimal gene targeting. In contrast, activation of MR persists over longer periods and between pulses (de Kloet, et al. 2005). Considerable heterogeneity exists in sensitivity of GR to their ligand between individuals (Cain and Cidlowski 2015) which may predict susceptibility to detrimental consequences of dysregulated circulating cortisol (Walker, et al. 1996).

The location and function of MR has long been thought to primarily be renal control of salt regulation and blood pressure control (Funder 2005). This has been challenged with more widespread identification of MR in non–epithelial tissue such as adipose. With the observation that activation of MR promotes differentiation of fibroblasts into mature adipocytes (Caprio, et al. 2007; Rondinone, et al. 1993), questions are raised as to whether the detrimental effects of glucorticoids on the development of obesity and features of the metabolic syndrome are in fact through stimulation of GR or MR. Certainly adipogenesis of human preadipocytes can be inhibited by MR antagonism (Caprio, et al. 2011) and glucose dysregulation in mice fed on a high fat diet can be ameliorated with spironolactone treatment, a selective MR antagonist (Wada, et al. 2010). The mechanism of action is postulated to be through down-regulated transcription of proinflammatroy cytokines such as TNF-α and up-

regulation of adiponectin and peroxisome proliferator-activated receptor-γ (PPAR- γ) (Guo, et al. 2008; Hirata, et al. 2009).

1.6 Cortisol metabolism

With recognition that the hydroxyl form of the glucocorticoid is required for biological activity, the enzyme involved in converting cortisone to cortisol in peripheral tissues was identified as 11β-hydroxysteroid dehydrogenase. High levels of activity were initially observed both in liver and kidney (Burton 1965). Purification 20 years later revealed bidirectional activity (Lakshmi and Monder 1985a) and it wasn't until characterisation of the isozyme in placenta, now recognised as 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) (Brown, et al. 1993), that two distinct forms of the enzyme were recognised.

Cortisol and cortisone are metabolised by the 5β-reductase enzymes, found primarily in the liver, generating dihydro- forms namely dihydrocortisol and dihydrocortisone. Only cortisol is metabolised by the 5α-reductase enzyme (type 1) producing allo-dihydrocortisol. Further reduction proceeds through the 3α-hydroxysteroid dehydrogenases generating the corresponding tetrahydro- metabolites (tetrahydrocortisol (THF), allo-tetrahydrocortisol (aTHF) and tetrahydrocortisone (THE)) which account for the majority of metabolic clearance of cortisol in humans. Measurement of these metabolites in urine and calculation of tetrahydrocortisol to tetrahydrocortisone ratios (THF:THE and aTHF:THE) provides assessment of whole body 11βHSD1 and 2 activities. Such measurements are impacted by alterations in the 5α- and β-reductase enzymes (Palermo, et al. 1996). Studies have shown

conflicting effects of obesity on cortisol metabolism with increased 5-α reductase activity observed in a study of obese men (Stewart, et al. 1999) yet increased 5-β reductase activity in a study involving obese men and post-menopausal women (Andrew, et al. 1998).

Metabolism of cortisol by 11βHSD2 generates inactive cortisone. This process is vital to prevent inappropriate activation of MR in epithelial tissues reliant upon tightly controlled electrolyte reabsorption such as renal cortex and medulla, salivary gland, colonic epithelial cells and sigmoid and rectal colon. The placenta additionally contains 11βHSD2 preventing excess exposure of the developing fetus to excess glucocorticoids.

1.7 11βHSD1 biology

In accordance with its structure, 11βHSD is a member of the short chain alcohol dehydrogenase/ reducatase (SDR) family made up of 292 amino acids in humans. The hydrophobic N terminus binds to the inner endoplasmic reticulum whilst the catalytic active site within the lumen allows binding of substrate and co-substrate for proton transfer. Co-localised within the endoplasmic reticulum is hexose-6-phosphate dehydrogenase (H6PDH) which provides the co-substrates NADPH and NADP+ in return for the oxidation of glucose-6-phosphate (G6P) to 6-phosphogluconate (Lavery, et al. 2006). Cortisone has a ten-fold greater affinity for the binding site of the enzyme than cortisol (Stewart, et al. 1994).

1.7.1 11βHSD1 Directionality

Hydroxysteroid dehydrogenase enzymes in general terms are involved in the interconversion between intermediate and end product steroids as per the steroid pathway (Figure 1.3) requiring nicotinamide containing co-substrates for hydride transfer. Such reactions are theoretically reversible but directionality is dependent upon several factors including pH, structural conformation of the enzyme and cofactor concentration. With gross alterations in these an enzyme may appear to act in a unidirectional manner (Mizrachi and Auchus 2009). The more thermodynamically stable reactions involving the reduction of a ketone to hydroxyl additionally promote unidirectionality.

Purified enzyme from tissue homogenate initially revealed 11βHSD1 as a bidirectional enzyme (Lakshmi and Monder 1985b). Work with intact cells challenged this with cortisone displaying much greater affinity for the enzyme than cortisol (Low, et al. 1994). Since then 11βHSD1 has been regarded as a principally reductase enzyme *in vivo.* There are several reasons why this may not hold true.

In intact cells, a high NADPH to $NADP^+$ ratio promotes oxo-reductase enzymes to catalyse reductase reactions (De Sousa Peixoto, et al. 2008). In the situation of 11βHSD1, this ratio is influenced by the function of H6PDH since NADPH is unable to permeate into the endoplasmic reticulum (Chapman, et al. 2013). Loss of H6PDH function as a result of genetic mutations (Lavery, et al. 2008) or reduced availability within immature stromal cells (Bujalska, et al. 2002) results in lack of reductase activity. Whether tissue specific alteration in enzymatic action seen in relation to obesity and T2DM reflects disruption of coenzyme or cosubstrate supply is unknown.

Other SDR enzymes such as 17-hydroxydehydrogenase enzymes, which interconvert weak and potent sex steroids, function in a bidirectional manner (Khan, et al. 2004). Directionality of these enzymes is similarly dependent upon whether the cell is intact or homogenised, and availability of co-substrate. Activity is additionally altered by temperature and pH (Luu-The, et al. 1995). It is of interest when comparing these enzymes that those utilising the non phosphorylated coenzyme, NAD, tend to favour the oxidative reaction whilst those preferring NADPH, which is in turn more abundant, drive in a predominantly reductase direction. Comparatively, 11βHSD1 is an NADPH dependent enzyme whilst 11βHSD2 relies upon NAD^+.

1.7.2 11βHSD1 regulation

Initial studies using purified rat liver enzyme found 11βHSD1 to principally catalyse the dehydrogenase reaction (Lakshmi and Monder 1988). Despite the addition of the co-substrate NADPH and the presence of cortisone, no reductase activity was observed. Further *in vitro* experiments using a variety of cell types proved that activity in the intact cell adopts reductase directionality (Bujalska, et al. 1997; Jamieson, et al. 1995; Rajan, et al. 1996). These contrasting observations may represent response to alterations in availability of substrate, co-enzyme and as a result, co-substrate although this has never been proven. Despite circulating at concentrations five to ten times lower than cortisol, the majority of cortisone remains unbound from protein which may influence enzyme function *in vivo*.

Cytokines such as TNF alpha and IL-1 can upregulate 11βHSD1 activity (Friedberg, et al. 2003) and therefore may have a role to play in tissue specific dysregulation of 11βHSD1 associated with chronic inflammatory conditions such as obesity

(Qatanani and Lazar 2007). Circulating growth hormone concentrations can additionally alter 11βHSD1 activity but with an inverse relationship (Stewart, et al. 2001). Gender specific sex steroids increase 11βHSD1 activity whilst leptin has varying effects between men and women (Dieudonne, et al. 2006).

The effects of insulin on 11βHSD1 appear to be variable between individuals and tissues. 11βHSD1 activity has been observed to be upregulated in adipocytes treated with insulin *in vitro* in some (Balachandran, et al. 2008) but not all studies (Napolitano, et al. 1998). *In vivo*, acute elevation in circulating insulin using a hyperinsulinemic clamp increased whole body and adipose 11βHSD1 activity (Wake, et al. 2006). Data from a similar study showed a rapid yet short lived reduction in adipose 11βHSD1 activity in lean individuals during acute hyperinsulinemia which was not corroborated in obese individuals (Sandeep, et al. 2005). Mechanistically, insulin results in translocation of glucose transporters to the cell membrane increasing the availability of co-substrate for 11βHSD1 activity.

Hepatocytes cultured with insulin *in vitro* have been observed to have reduced 11βHSD1 mRNA but with little effect on overall activity (Jamieson et al. 1995). *In vivo* obese euglycaemic individuals display down regulated hepatic 11βHSD1 activity (Rask et al. 2001; Rask et al. 2002; Stewart et al. 1999) which is upregulated with the addition of relative insulin deficiency as seen with T2DM (Stimson, et al. 2011).

A study performed by Stimson et al. showed upregulation of 11βHSD1 activity in whole body in response to a high carbohydrate meal (Stimson et al. 2014). The magnitude of this response was in proportion to the increase in whole body

circulating insulin concentrations providing a potential causation which is backed up by findings that acute hyperinsulinaemia enhances 11βHSD1 reductase activity (Wake et al. 2006).

Glucocorticoids themselves have a positive feedback on 11βHSD1 upregulating tissue activity resulting in increased cortisol regeneration (Dube, et al. 2015b; Morgan, et al. 2014).

1.7.3 Transcription and modification of 11βHSD1

The human gene encoding 11βHSD1 (*HSD11B1*) is located on chromosome 1 (1q32-41) encompassing 6 exons. The levels of transcripts have been shown to directly correlate with enzyme activity (Lindsay et al. 2003; Wake et al. 2003). Post-translational modification of the protein may alter function and directionality although this remains controversial. Agarwal et al. have observed that glycosylation of rat 11βHSD1 was necessary for maximal dehydrogenase activity (Agarwal, et al. 1990). Further work by the same group went on to investigate this further by creating loci directed mutagenesis at two individual glycosylation sites. Mutation of one of these sites showed a reduction in both dehydrogenase and reductase activity. Mutation of both glycosylation sites compounded the effect resulting in negligible reductase or dehydrogenase activity (Agarwal, et al. 1995). Conflicted results have been observed using human liver 11βHSD1 where non-glycosylated forms of the enzyme retain dehydrogenase and reductase activity (Blum, et al. 2000; Walker, et al. 2001).

1.7.4 Genetic variation in human *HSD11B1*

The impact of reduced 11βHSD1 reductase activity is observed with the condition of Cortisone Reductase Deficiency (CRD). With relatively greater peripheral clearance of cortisol, HPA axis stimulation drives increased steroid synthesis which is most apparent through elevated androgen concentrations (testosterone, androstenedione and dehydroepiandrosterone sulphate (DHEAS)). This presents itself in boys as pseudo-puberty whilst in females results in infertitlity, acne and hirsutism. Measurement of urinary metabolites in such individuals have revealed a twenty-five fold reduction in the ratio of cortisol: cortisone urinary metabolites (Phillipov, et al. 1996). Initial association studies failed to correlate *HSD11B1* gene polymorphisms with identified cases (Jamieson, et al. 1999). With 11βHSD1 function relying on the generation of co-substrate, genetic studies moved focus to the coenzyme H6PDH revealing mutations in four separate cases (Lavery et al. 2008). More recent work has, however, now observed heterozygous mutations in the coding region of *HSD11B1* returning the concept of direct as opposed to apparent cortisone reductase deficiency (Lawson, et al. 2011).

1.8 Measurement of 11βHSD1 activity

Quantification of urinary cortisol and cortisone metabolites has been used for the assessment of disordered whole body 11βHSD activity (Phillipou and Higgins 1985; Wilson, et al. 1995). This classically involves calculation of ratio of cortisol (urinary free cortisol, THF and allo-THF) to cortisone metabolites (urinary free cortisone and THE). This does not allow for distinction between metabolism by the 11βHSD enzymes and 5 α-reductase enzyme. Seperation of conjugated (THF, allo-THF and

THE) from unconjugated (cortisol and cortisone) metabolites can allow distinction of enzyme activity between kidney and liver (Best and Walker 1997) but does not allow quantification of activity in alternative tissues such as adipose or muscle and does not allow quantification of unidirectional activity of the 11βHSD enzymes (Walker, et al. 1993).

With evidence of tissue specific dysregulation of cortisol with obesity (Rask et al. 2001; Stewart et al. 1999), the need for selective measurement of reductase and dehydrogenase activity by 11βHSD in whole body and across tissue becomes apparent. Although this may be provided by glucocorticoid quantification in tissue biopsies, this is not always practical and does not allow assessment of enzyme response to acute perturbations. With a bioavailability of 96%, oral cortisone is rapidly converted to its active form through first pass metabolism predominantly through the action of the liver (Derendorf, et al. 1991) and more specifically through 11βHSD1 (Andrew, et al. 2005). By measuring the rate of appearance of cortisol following oral cortisone ingestion, reductase activity by hepatic 11βHSD1 can be quantified.

Tracers have been used since the mid 1900s to track metabolic pathways in the body. Radioactive compounds were some of the first to be used. Following infusion, enzyme activity can be quantified by measuring the difference in radioactivity between influx and efflux. More current methods involve the use of isotopes *i.e.* two compounds with the same atomic number but different atomic mass (the number of protons and neutrons). Isotopologues occur naturally although at much smaller quantities than their parent compound. For use as a tracer these therefore often

require synthetic generation based on an endogenous compound, known as the tracee. Enzyme activity is calculated by measuring the dilution of one to the other referred to as the tracer to tracee ratio. Due to the potential harmful effects of using radioactive tracers and the ability to quantify increasingly smaller concentrations of endogenous compounds with greater specificity, isotopic tracers are now more commonly used. Such tracers have allowed 11βHSD1 activity to be quantified in whole body and across tissues including adipose (Stimson, et al. 2009), liver (Basu, et al. 2009; Stimson et al. 2011) and skeletal muscle (Basu, et al. 2004).

1.8.1 11βHSD1 reductase activity

By replacing four hydrogen atoms with deuterium, the stable isotope of cortisol, 9,11,12,12-[^{2}H]$_{4}$–cortisol (D4-cortisol) is produced. In keeping with the definition of a tracer, this behaves chemically and physiologically like the endogenous compound. It is only the mass difference which allows distinction and quantification. The deuterium atom attached to carbon at position 11 is removed by dehydrogenation generating 9,12,12-[^{2}H]$_{3}$-cortisone (D3-cortisone). With the natural abundance of deuterium being less than 0.02%, this is an irreversible reaction. Further reduction is specific to 11βHSD1 generating 9,12,12-[^{2}H]$_{3}$-cortisol (D3-cortisol) (Figure 1.5). The rate of appearance of D3-cortisol on the background of steady influx of D4-cortisol allows the specific reductase activity of 11βHSD1 to be calculated.

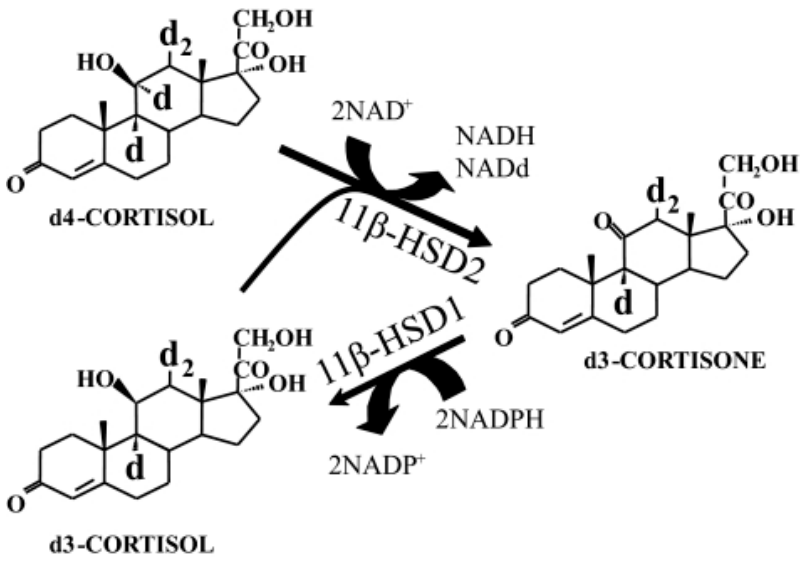


Figure 1.5 Schematic showing the use of deuterated tracer D4-cortisol to quantify cortisol to cortisone interconversion by 11βHSD1 and 11βHSD2

1.8.2 11βHSD1 dehydrogenase activity

More recent work within our department has validated the use of a deuterated tracer for the measurement of dehydrogenase activity by the 11βHSD enzymes (Hughes, et al. 2012). D2-cortisone (1,2-[^{2}H]$_{2}$-cortisone) (Figure 1.6) is infused to achieve steady state and the rate of dilution by endogenous cortisone provides a measure of dehydrogenase activity by the 11βHSD enzymes.

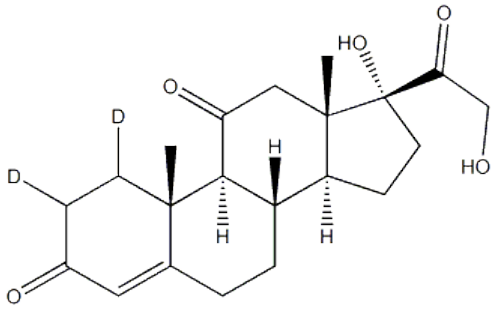


Figure 1.6 Schematic showing deuterated tracer D2-cortisone used for the measurement of dehydrogenase activity by 11βHSD1 and 11βHSD2

1.8.3 Tissue specific measurement of 11βHSD1 activity

Tracers have allowed quantification of adrenal cortisol synthesis and tissue cortisol production rates by 11βHSD1. Cortisol regeneration in splanchnic tissue has been found to equate that of the adrenal gland (Andrew et al. 2005) and is almost entirely accounted for by the liver (Basu et al. 2009; Stimson et al. 2009).

The first evidence of tissue specific dysregulation of cortisol in the setting of obesity dates back to studies performed by Per Bjorntorp (Marin et al. 1992) who revealed an inverse relationship between measures of central obesity and early morning cortisol concentrations as a result of higher excretion of cortisol metabolites, mainly in the form of cortisone, in urine. Despite lean and obese individuals having similar whole body 11βHSD1 activity, obese individuals display upregulated activity in adipose tissue (Rask et al. 2001; Sandeep et al. 2005; Stimson et al. 2009). Hepatic 11βHSD1 activity has been observed to be lower in obese individuals (Rask et al. 2001; Rask et al. 2002; Stewart et al. 1999). This counter-balance is disrupted in the setting of T2DM where hepatic enzyme activity is sustained (Stimson et al. 2011) hypothesised

to be the result of relative insulin deficiency. This latter finding has been challenged by another study (Basu, et al. 2005).

Deuterated cortisol tracers have been used to investigate acute perturbation in 11βHSD1 activity with evidence that acute elevation in insulin increase whole body 11βHSD1 *in vivo* (Stimson, et al. 2014). Across individual tissues, elevated insulin and free fatty acid concentrations increases enzyme activity (Wake et al. 2006). This effect may not be consistent between lean and obese individuals (Sandeep et al. 2005).

1.9 Tissue regulation of 11βHSD1

Distribution of 11βHSD1 is diffuse in metabolically active tissues throughout the body. By polymerase chain reaction, 11βHSD1 RNA has been located in the liver, lung spleen, cerebellum, pituitary, kidney medulla, and placenta (Whorwood, et al. 1995).

1.9.1 Splanchnic tissue

It was from rat liver tissue that purification and characterization of 11βHSD1 was first detailed (Lakshmi and Monder 1985b, 1988) and is now recognized as having greatest enzyme expression in both rats and humans (Tannin, et al. 1991). This has been corroborated by activity studies which have shown that liver accounts for 11βHSD1 within splanchnic tissue almost entirely (Basu et al. 2009; Stimson et al. 2009) and in turn generates cortisol at equivalent rates to that of the adrenal gland (Andrew et al. 2005). Enzyme activity has been observed to be nine fold greater in the liver compared with visceral adipose tissue (Basu et al. 2009). The significance

of cortisol regeneration by the liver is additionally exemplified in liver specific 1βHSD1 knockout mice with the development of adrenal hyperplasia in response to chronic activation of the HPA axis (Lavery, et al. 2012).

In mouse models of T2DM, a positive correlation has been observed between circulating glucose concentrations and hepatic 11βHSD1 mRNA and activity implicating this tissue as a potential driver for development of glucose dysregulation associated with obesity (Liu, et al. 2005). Using genetically modified knockout mice the mechanism of action has been postulated to be through key gluconeogenic enzymes in the liver including glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK) (Kotelevtsev, et al. 1997). Whole body 11βHSD1 knock-out mice have been observed to have more favourable lipid profile (Morton, et al. 2001) through altered regulation of lipolytic enzymes (Morton, et al. 2001). The more recent development of liver specific knockout mice has, however, failed to corroborate such beneficial effects (Lavery et al. 2012) implying potential cross talk between target organs or perhaps compensatory mechanisms by the HPA axis (la Fleur, et al. 2005).

Hepatic 11βHSD1 has a further role to play in catalysing the inter-conversion between bile acids (Odermatt, et al. 2011) which in turn have an inhibitory effect on 11βHSD enzyme activity (Diederich, et al. 2000). Their role in altering enzyme activity with obesity and T2DM are unknown.

1.9.2 Adipose tissue

Some studies have shown the sole presence of 11βHSD1 in *ex vivo* adipose tissue whilst others have additionally observed 11βHSD2 (Bujalska, et al. 1999; Engeli, et

al. 2004; Lee, et al. 2008). With distinctly different embryological origins (Chau, et al. 2014) it has been debated whether subcutaneous or omental adipose tissue is more influential in the development of adverse metabolic outcome. *Ex vivo* adipose tissue samples from men and women undergoing abdominal surgery showed greater reductase activity in omental compared with subcutanous tissue (Bujalska et al. 1997). These results have not been confirmed in a further cohort of women (Veilleux, et al. 2010). 11βHSD1 activity in subcutaneous adipose is positively correlated with obesity in men (Rask et al. 2001) and women (Rask et al. 2002). Increased 11βHSD1 activity in subcutaneous adipose tissue is thought to potentiate adverse metabolic consequences to a greater degree than that in omental adipose tissue (Alberti, et al. 2007) and positively correlates with fasting plasma glucose, plasma insulin concentrations and insulin resistance (Lindsay, et al. 2003). A recent study using microdialysis found upregulated 11βHSD1 activity in obese individuals with T2DM but not in obese euglycaemic individuals (Dube, et al. 2015a).

1.9.3 Skeletal muscle

The effect of glucocorticoids on muscle is well understood with induction of atrophy, protein breakdown (Biedasek, et al. 2011; Löfberg, et al. 2002) and impairment of insulin induced glucose uptake (Morgan, et al. 2009). Skeletal muscle accounts for the major site of glucose disposal and as such is particularly vulnerable to the inhibiting effects of cortisol on insulin function (DeFronzo, et al.).

With a negative correlation between myocyte 11βHSD1 and glucose disposal rate as well as a positive correlation with BMI and systolic blood pressure, it is postulated that enzyme activity in skeletal muscle may play a significant role in the development of the metabolic syndrome (Whorwood, et al. 2002). In agreement,

myotubes from obese patients with type 2 diabetes mellitus (T2DM) have been shown to have a two fold increase in 11βHSD1 mRNA compared with control individuals (Abdallah, et al. 2005). This finding has not been consistent (Jang, et al. 2007).

Localisation of 11βHSD2 in skeletal muscle has been conflicting (Abdallah et al. 2005; Hassan-Smith, et al. 2015; Jang et al. 2007; Whorwood et al. 2002) but may rely upon sensitivity of the gene transcription technique used. A recent finding of mineralocortcoid receptors in human skeletal muscle (Chadwick, et al. 2015) provides a potential physiological need for the presence of 11βHSD2 in this tissue.

Despite excess abundance of co-enzyme H6PDH for regeneration of co-substrate NADPH, 11βHSD1 mRNA is not directly correlated with reductase activity in skeletal muscle (Jang, et al. 2006; Jang et al. 2007). Alternative directionality of the enzyme or inhibition of enzyme activity as a result of local factors could be potential explanations. It has, for instance, been observed in *ex vivo* muscle biopsies that 11βHSD1 reductase activity is downregulated in individuals with T2DM (Jang et al. 2007) thought to be the result of relative insulin deficiency. Incubation with dexamethasone reverses this effect returning enzyme activity to that of lean control individuals. In contrast, 11βHSD2 activity appears resistant to this effect (Whorwood et al. 2002). Although the contribution of muscle cortisol regeneration is likely to be small in comparison to splanchnic tissue, it may confer some protective effect against the metabolic sequelae associated with the metabolic syndrome. Indeed, inhibitors of 11βHSD1 are likely to improve insulin sensitivity in part through their effect on

skeletal muscle (Morgan et al. 2009). Few studies have, however, detailed skeletal muscle 11βHSD1 activity in humans *in vivo*.

1.10 Preclinical models of manipulation of 11βHSD1

The first evidence of improved metabolic consequences of 11βHSD1 inhibition came through whole body 11βHSD1 knock out in genetically modified mice. These animals resisted hyperglycaemia despite being fed on a high fat diet or being exposed to environmental stress (Kotelevtsev et al. 1997). Work has since been undertaken to try to localize the tissue or tissues responsible for this overall effect. Genetically modified mice over expressing 11βHSD1 in adipose tissue (Masuzaki, et al. 2001) revealed elevated glucocorticoid levels within adipose tissue yet unaltered circulating concentrations, mirroring findings in human obesity. The phenotype was additionally consistent with the metabolic syndrome with glucose dysregulation, hypertension and dyslipidaemia. Mice over expressing 11βHSD1 selectively in the liver developed similar adverse metabolic features of glucose dysregulation but lacked the development of obesity (Paterson, et al. 2004). Liver specific knockout mice have additionally been generated which displayed mild improvement in glucose tolerance but limited effect on insulin sensitivity, adposity and weight (Lavery et al. 2012). These limited improvements in metabolic phenotype were disappointing in light of the knowledge of the significant contribution of liver to whole body cortisol regeneration by 11βHSD1 (Andrew et al. 2005; Basu et al. 2009). Further investigations have provided more encouraging results from liver specific knock down mice resisting the development of hyperinsulinaemia and display normal

glucose tolerance despite treatment with glucocorticoids (Harno, et al. 2013). It is questionable whether this may be mediated by altered clearance of administered steroids. By comparing the effects of adipose specific and liver specific 11βHSD1 knockout mice it is probable that dyslipidaemic effects of glucocorticoids are directed from adipose tissue whilst the development of glucose dysregulation is driven by the liver (Morgan et al. 2014).

1.11 11βHSD1 inhibitors

The concept of pharmacological inhibition for the treatment of the metabolic syndrome has been proven in genetically modified hyperglycaemic hyperinsulinaemic murine models with resultant reduction in endogenous glucose production, circulating insulin concentrations and improvement in dyslipidaemia (Alberts, et al. 2002). Studies have since progressed to human clinical studies.

1.11.1 Non-selective inhibition

Carbenoxolone was the first 11βHSD inhibitor to be trialled in humans. With evidence of increased whole body insulin sensitisation in healthy volunteers (Walker, et al. 1995), carbenoxolone was trialled in men with T2DM (Andrews, et al. 2003). After 7 days there was no effect on the rate of glucose disposal although the rate of glycogenolysis during hyperglucagonaemia was signficantly reduced. As would be anticipated blood pressure rose due to the non-selective action of carbenoxolone on the isozyme 11βHSD2. Future development of carbenoxolone as treatment for T2DM was limited after failure to show an improvement on glycosylated

haemoglobin (HbA1c) and with known adverse effects on blood pressure (Sandeep, et al. 2004).

1.11.2 Selective inhibition

The first selective 11βHSD1 inhibtor for clinical use was developed by Biovitrum with a thirty-fold higher selectivity for the type 1 isozyme (Alberts et al. 2002). In hyperglycaemic and hyperinsulinaemic mice, 7 days treatment resulted in reduced circulating glucose and insulin levels but with little impact on food intake and weight. Collaboration with Amgen led to the development of AMG-311 which entered phase II trials but in 2005 was discontinued (Jones RM 2012). With lack of success in clinical studies from whole body 11βHSD1 inhibition, work progressed targeting enzyme activity in adipose tissue. Biovitrum's later compound, BVT-116429 resulted in a 90% reduction in 11βHSD1 activity within adipose tissue 6 hours post dose in mice (St. Jean, et al. 2007). In diabetes induced (KKAy) mice and in human adipocytes *in vitro*, tissue specificity was shown through the unique effect of this compound in elevating adiponectin production. After seven days treatment, basal insulin levels in mice were reduced but there was no improvement in insulin sensitivity. The limitation of adipose tissue specific inhibitors was apparent with a lack of reduction in hepatic gluconeogenesis independent of any alteration in fat mass or circulating lipids (Sundbom, et al. 2008).

1.11.3 Phase 2 trial data for Type 2 Diabetes Mellitus

Both Merck and Incyte have developed compounds which have reached phase II trials (Table 1.1) with beneficial effects on glycaemic control. Incyte's compound INCB-13739 showed lipid lowering effects and increased insulin sensitivity in a phase I trial (Hawkins, et al. 2008) and reduction in HbA1c by 5.5 mmol/mol after

12 weeks in phase II trial (Rosenstock, et al. 2010). Improvement in HbA1c was greatest in those with poorest control and largest BMI at baseline supporting the need for appropriate patient selection.

The concept of 11βHSD1 inhibitors as novel agents for the treatment of T2DM is evident with potential favourable cardiovascular risk reduction through alteration in lipids, blood pressure and body weight. The maximum benefit may require longer administration than the currently studied 12 week drug exposure masking their full potential. Pfizer validated pharmacodynamic biomarkers of 11βHSD1 inhibition *in vivo* through the use of urinary cortisol metabolites and the conversion of oral prednisone to prednisolone (Courtney, et al. 2008). Using gold standard tracer technique, the target effect of 11βHSD1 inhibition has been assessed for only one compound, AB-384. Using a primed continuous D4-cortisol infusion, enyme activity was suppressed *in vivo* by over 80% across whole body (Katz, et al. 2013).

Pharmaceutical agent and study details	Primary outcome	Secondary outcomes	Other effects
Rosenstock et al. Diabetes Care 2010 (Rosenstock et al. 2010) INCB-13739 302 patients with type 2 diabetes (mean BMI 32kg/m^2, mean HbA1c 8.6%) on metformin Randomised to one of 6 doses INCB-13739 or placebo for 12 weeks	Reduced HbA1c (-0.56%)	Reduced fasting plasma glucose (-1.3mM) Improved HOMA-IR (24%) Reduced body weight (-1kg) Reduced total cholesterol (-3%) No change in BP	Plasma cortisol and salivary cortisol unaltered Elevated ACTH (100%), DHEAS (54%) and androstenedione (60%) In females elevated total testosterone but not FAI
Feig et al. Diabetes, Obesity Metabolism 2011(Feig, et al. 2011) MK-0916 154 patients with type 2 diabetes (mean BMI 32kg/m^2, mean HbA1c 7.3%) with $\geq$ 1 metabolic syndrome features Randomised to one of 3 doses MK-0916 or placebo for 12 weeks	No effect on fasting plasma glucose	Reduced HbA1c (-0.3%) No effect on post-prandial glucose Reduced body weight (-1.8kg) with a trend for waist reduction Reduced BP (-8/5 mmHg) Increase LDL cholesterol (10%)	Elevated DHEA (33%), androstenedione (22%) and testosterone (26%) No reported hirsutism or acne

Shah et al. J Am Soc Hypertension (Shah, et al. 2011) MK-0916 and MK-0736 Hypertensive non-diabetic patients with BMI 27 – 41kg/m^2 (n= >50/group) or 20- 27kg/m^2 (n = 19/group) Randomised to one of 2 doses of MK-0736, one dose of MK-0916 or placebo for 12 weeks	Non significant reduction in sitting BP	Reduction in daytime BP (-6/2 mmHg) Weight reduction (-1kg) Reduction LDL cholesterol (- <5%)	Elevated DHEA (54%), androstenedione (42%), testosterone (57%) No effect hirsutism or acne
Freude et al. Diabetes, Obesity and Metabolism (Freude, et al. 2016) BI 135585 72 men and post-menopausal women with T2DM with BMI 25-40 kg/m^2 and HbA1c <8.5% Randomised to one of six doses of BI 135585 or placebo for 14 days	Enzyme inhibition (65-75%) across liver (assessed using THF/THE ratio in urine). No enzyme inhibition in *ex vivo* adipose tissue samples after 14 days treatment	Plasma glucose, HbA1c unchanged after 14 days	Increased ACTH concentration and increased urinary cortisol metabolites. Increased DHEAS and androstenedione concentrations although majority still within the reference range and returned t baseline 10 days after discontinuation

Heise et al. Diabetes, Obesity and Metabolism (Heise, et al. 2014) RO-151 and RO-838 110 men and women with T2DM BMI 26-42 kg/m^2 and HbA1c between 7 and 10% on metformin treatment Randomised to 10 or 400 mg RO-151, 50 or 200 mg RO-838 or placebo in combination with netformin for 28 days	No effect on mean daily plasma glucose or fasting plasma glucose concentrations	Reduction in HbA1c with RO-151 (-0.37%) Dose dependent reduction in weight across all groups (between -0.86 to -1.67 kg) Increase in trigylcerides (40%) and VLDL (25%) with high dose RO-838	Increase SBP (1.4-5.1 mmHg) and DBP (1.5-4.5 mmHg) with high dose RO-838 Reduction in SBP (mean -3.53 mmHg) with low dose RO-151 Dose dependent elevation in ACTH with RO-151 (44-69%) Increase DHEA, androstenedione with RO-151

Table 1.1 Results of published Phase II trials with selective 11βHSD 1 inhibitors for treatment of type 2 diabetes mellitus

ACTH; adrenocorticotropic hormone; BMI: body mass index; BP: blood pressure; DBP: diastolic blood pressure; DHEA: dehydroepiandrosterone; DHEAS: dehydroepiandrosterone sulphate; FAI: free androgen index; HbA1c: glycosylated haemoglobin; LDL: low density lipoprotein; SBP: systolic blood pressure; THF: tetra-hydrocortisol; THE: tetra-hydrocortisone

1.11.4 Additional therapeutic effects of 11βHSD1 inhibition

Cortisol dysregulation through the action 11βHSD1 has been implicated in the development of conditions associated with the metabolic syndrome and T2DM. Pre-clinical studies have shown that 11βHSD1 inhibition prevents progression of atheromatous plaque disease and reduces deposition of cholesterol in the aorta (Atalar, et al. 2012; Hermanowski-Vosatka, et al. 2005). Such findings have not been replicated in humans although elevated 11βHSD1 in the ascending aorta of individuals with the metabolic syndrome and concomitant coronary artery disease provides circumstantial evidence for extrapolation from mice (Atalar et al. 2012). These beneficial effects on neointimal proliferation can also be utilised in the setting of peripheral artery atheromatous disease as has been shown in atherosclerotic prone Apo E knockout mice fed on a high fat western diet (Iqbal, et al. 2012). Since these findings were not replicated in the same study using C57B16 mice the mechanism of action may be local in the vessel wall or through systemic risk factors such as blood pressure.

As well as their proliferative effect on intimal smooth muscle, glucocortcoids play a role in the inhibition of angiogenesis at sites of tissue ischaemia. Mice with global 11βHSD1 deficiency have exaggerated angiogenesis in skin wounds following subcutaneous sponge insertion, and in myocardium following coronary artery ligation (Small, et al. 2005). This has been associated with protection from heart failure following myocardial infarction in mice (McSweeney, et al. 2010). If replicated using pharmacological 11βHSD1 inhibitors, this may provide additional

benefits to treating patients with T2DM post cardiovascular event or those with chronic foot ulcerations.

Deletion or inhibition of 11βHSD1 has been observed to reduce age related cognitive dysfunction in mice (Sooy, et al. 2010; Yau, et al. 2001). In human clinical studies the non selective inhibitor carbenoxolne improved memory in individuals with and without T2DM (Sandeep et al. 2004). More recently Abbott developed a selective 11βHSD1 inhibitor which reached phase II clinical trial. The primary outcome was change from baseline in cognitive function and memory as assessed by Alzheimer's Disease Assessment Scale (ADAS). Neither dose trialed resulted in improvement of cognitive function compared to placebo, but it appears the compound did not penetrate central nervous system sufficiently to achieve adequate inhibition of brain 11βHSD1. Preclinical studies have provided evidence of efficacy in target enzyme inhibition (Liu, et al. 2013) and 11βHSD1 inhibition in a mouse model of Alzheimer's disease prevented cognitive decline with chronic treatment (Sooy, et al. 2015).

Patients with T2DM are at increased risk of developing nonalcoholic steatohepatitis with potential development of liver fibrosis and cirrhosis. Hyperactivity of the HPA axis has been observed in the setting of non-alcoholic fatty liver disease (NAFLD) implicating subclinical hypercortisolism as the pathogenic mechanism (Targher, et al. 2006). Roche have pursed this with the progression of one of their compounds to phase II trial for the treatment of NAFLD (Stefan, et al. 2014). Twelve weeks treatment resulted in a significant reduction in liver fat content in overweight (BMI > 27 kg/m^2) individuals with compounding insulin resistance.

1.11.5 Toxicity of 11βHSD1 inhibitors

In general 11βHSD1 inhibitors have been well tolerated over 12 week's administration in clinical studies. Concerns remain about potential effects on whole body homeostasis with their use through alteration of HPA axis. With reduced tissue glucocorticoid synthesis, net cortisol clearance is increased. As a result a compensatory upregulation of the HPA axis is likely to ensue through stimulation of CRH release and ACTH production. Studies have confirmed that this maintains whole body circulating cortisol (Hawkins et al. 2008; Rosenstock et al. 2010) but at the expense of activating other steroidogenesis pathways, notably adrenal androgens. Whether this effect on steroidogenesis is of clinical significance remains unclear. The greatest impact is likely to be in female patients resulting in hirsutism, acne and menstrual irregularities. Such effects have been shown to be reversible upon withdrawal (Feig et al. 2011) although prescription is likely to be avoided in females of child bearing age.

Concern has been raised whether reducing peripheral cortisol concentrations may impair the body's ability to respond to stress, infection and inflammation. Target individuals with poorly controlled T2DM are already at higher risk of infection which could be compounded by such agents. Any increased incidence of infective symptoms in studies to date has been mild and localized (Courtney et al. 2008). With co-administration of hypoglycaemic agents the risk of developing and the response to hypoglycaemia may be impaired which would require close monitoring (Harno and White 2010). With structural homology, neuroactive steroids and oxysterols are metabolized by 11βHSD1. Whole body inhibition may therefore have resultant

consequences on neurotransmission and bile acid synthesis (Odermatt and Nashev 2010).

1.11.6 Questions for the efficacy of 11βHSD1 inhibition

With promising results from *in vitro* data (Schweizer, et al. 2003), it is of interest to consider whether post transcriptional regulation of 11βHSD1 is different *in vivo* in diabetic individuals or whether other factors may be masking their full effect. With few published clinical studies recruiting diabetic patients much remains uncertain about 11βHSD1 activity in the context of hyperglycaemia and its treatment.

As a novel agents for the treatment of T2DM it is likely that their prescription will be second or third line alongside well established therapies for diabetes such as metformin or gliclazide.

Metformin has been available for over fifty years and is the most frequently prescribed anti-diabetic treatment worldwide. Its popularity stems from additional benefits such as reduction in caloric intake (Glueck, et al. 2001), hypothesized to be due to increased transcription of the incretin hormone, glucagon-like peptide 1 (GLP-1) (Maida, et al. 2011), with consequential reduction in weight (Salpeter, et al. 2008). Moreover, this weight reduction has been observed to be predominantly due to reduction in adipose tissue rather than muscle mass (Stumvoll , et al. 1995). Evidence for beneficial effects on cardiovascular risk factors such as blood pressure (Giugliano, et al. 1993; Hermann, et al. 1994) and circulating lipid levels (Hermann et al. 1994; Salpeter et al. 2008) as well as hard end-point cardiovascular disease (Boussageon, et al. 2012; Hemmingsen, et al. 2012; UKPDS 1998) has been disputed between studies.

The glucose lowering effect of metformin is thought to be through inhibition of gluconeogenesis by the liver (Shaw, et al. 2005) and increased glucose disposal into skeletal muscle (McIntyre, et al. 1991). The principle cellular membrane transporters of metformin, solute carrier family 22 members (SLC22A), are highly expressed in the liver where the effects of metformin are thought to predominate. By increasing activation of insulin receptor and insulin receptor substrate (IRS-2) as well as promoting translocation of glucose transporters (Gunton, et al. 2003), metformin is thought to improve cellular sensitivity to insulin with resultant suppression of gluconeogenic pathways. The gluconeogenic effect of glucagon is additionally blocked by metformin further reducing circulating glucose concentrations (Miller, et al. 2013). All of this results in improved utilization of glucose and as a result reduction in the requirement of insulin secretion.

At a cellular level, metformin inhibits complex 1 of the mitochondrial electron transport chain (Pernicova and Korbonits 2014) causing a reduction in adenosine triphosphate (ATP) and increase in adenosine monophosphate (AMP) production. This drop in ATP inhibits the action of glycogen in synthesizing cyclic adenosine monophosphate (cAMP) and drives the action of 5'-AMP-activated protein kinase (AMPK). AMPK acts as a cellular 'fuel gauge' driving catabolic pathways, such as fatty acid oxidation (Merrill, et al. 1997) and switching off ATP consuming pathways such as gluconeogenesis. AMPK additionally increases the activity of insulin receptor function improving tissue sensitivity and stimulates the migration of glucose transporters to the cell membrane (Gunton et al. 2003).

The involvement of AMPK in the mechanism of action of metformin has been questioned. Using AMPK deficient murine models, metformin has been observed to disrupt hepatic gluconeogenic pathways in an AMPK- and transcription-independent manner (Foretz, et al. 2010). In accordance with this a further study showed a direct effect of metfornin on increasing AMP, without the involvement of AMPK, thereby antagonizing the effect of glucagon on cAMP and resulting in reduced activation of protein kinase A. Down-stream kinases fail to be phosphorylated disrupting gluconeogenic pathways involving phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (Miller et al. 2013).

In contrast, the mechanism of action of alternative oral hypoglycaeic agents such as gliclazide is well detailed. Gliclazide is thought to stimulate insulin secretion through activation of the sulphonylurea receptor on pancreatic β cells. It may have addition beneficial effects on hepatic glucose production and post insulin receptor signaling (Campbell, et al. 1991a).

With the knowledge of tissue specific dysregulation of 11βHSD1 in the setting of T2DM, it is postulated whether 11βHSD1 inhibitors may be more efficacious if they target specific tissues. As discussed previously (Section 1.10), knockout of the enzyme in liver in murine models showed some improvement in glucose tolerance but with limited benefit on weight reduction. No genetically modified models of adipose specific 11βHSD1 knockout have been generated. Biovitrum's compound, BVT-116429 was observed to have high availability in adipose tissue displaying 90% reduction in 11βHSD1 6 hours post dose in male mice (St. Jean et al. 2007). In a diabetic KKA^y mouse model, this agent reduced fasting glucose after 10 days

treatment (Sundbom et al. 2008). Adiponectin levels increased suggesting adipose-specific mechanism of action. Disappointingly body weight remained steady with no significant reduction in total body adiposity. Additionally, although basal insulin levels were reduced, an insulin tolerance test did not show any significant improvement in sensitivity, although this was only over a 10 day treatment period. 11βHSD1 inhibition has been observed using the same compound in cultured primary human adipocytes *in vitro* although it has not progressed to phase 2 trials. There is therefore as yet no strong evidence for improved efficacy with tissue specific inhibition of 11βHSD1 for the treatment on T2DM.

Early experiments observed 11βHSD1 to be a dehydrogenase enzyme in experiments in homogenised rat liver (Lakshmi and Monder 1988). Further work using intact cells revealed predominant reductase activity (Jamieson et al. 1995). These conflicting results were thought to be due to altered availability of co-substrate but this has never been proven. Bidirectional activity was observed *in vivo* in adipose tissue through the use of cortisol and cortisone tracers (Wake et al. 2006). Despite this it has been suggested that 11βHSD1 acts solely as a reductase enzyme *in vivo* and dehydrogenase enzyme *in vitro*. It is unquestionable that in metabolically active tissue 11βHSD1 acts primarily as a reductase enzyme. Whether this is influenced by whole body glucocorticoid dysregulation such as with HPA axis alteration in the presence of the metabolic syndrome (Reynolds et al. 2001a) is unknown. Additionally we have observed tissue specific 11βHSD1 reductase activity in relation to obesity and T2DM but we do not know the impact on corresponding dehydrogenase activity.

Work undertaken within our department has confirmed bidirectional 11βHSD1 activity in adipose tissue and skeletal muscle in healthy male volunteers (Hughes et al. 2012). Across splanchnic tissue, there was a preponderance of reductase activity at a ratio of 3:1. In adipose tissue there was a slight shift towards dehydrogenase activity (reductase: dehydrogenase; 1:1.3) with equal rates across skeletal muscle. It can be hypothesised that at a cellular level, cortisol regulation may exist through an ability to regulate these opposing reactions. A greater understanding of regulation of cortisol at an intracellular level may allow the development of more efficacious pharmacological agents for the treatment of T2DM and coexisting features of the metabolic syndrome.

1.12 Hypothesis

(a) Metformin downregulates 11βHSD1 activity masking the efficacy 11βHSD1 inhibitors for the treatment of type 2 diabetes mellitus

(b) Measurement of total cortisol (free plus bound) underestimates the contribution of 11βHSD1 activity to the circulating plasma pool

(c) 11βHSD1 acts as a bidirectional enzyme *in vitro* and *in vivo*

(d) 11βHSD1 acts predominately as a reductase enzyme in obese individuals which is further enhanced by acute perturbations in circulating insulin concentrations

1.13 Aims

This thesis seeks to investigate the control of intracellular cortisol and cortisone through the action of 11βHSD1 particularly focusing on

(a) whether metformin treatment acts through 11βHSD1 to regulate cellular cortisol obscuring the efficacy of inhibitors

(b) whether clinical studies have underestimated the contribution of 11βHSD1 to the circulating pool by addressing total rather than free hormone and

(c) the impact of recycling between cortisol and cortisone in whole body, adipose tissue and skeletal muscle between lean and obese individuals

Chapter 2: Materials and methods

2.1 Introduction to materials and methods

All measurements and samples collected during clinical studies were undertaken by the author except those declared in Section 3.4. Assays in routine departmental use were optimised as described below for analysis of deuterated tracers as well as endogenous glucocorticoids in particular for measurement in human plasma and cell culture medium. Where stated, room temperature corresponds to a temperature of 18-22 ºC.

2.2 Equipment

2.2.1 Laboratory equipment

2.2.1.1 Centrifuges

2.2.1.1.1 Clinical Studies

Eppendorf Centrifuge 5810 R (Eppendorf AG, Hamburg, Germany).

2.2.1.1.2 Free cortisol method development

Heraeus Sepatech Biofuge 13 centrifuge (Heraeus, Hanau, Germany).

2.2.1.1.3 Cell Culture

Heraeus Labofuge 400 R Centrifuge (Heraeus, Hanau, Germany).

2.2.1.2 pH meter

Thermo Orion 410 A (Fisher Scientific, Loughborough, UK).

2.2.1.3 Plate rotator

iEMS incubator/shaker (Thermo Scientific Inc, Waltham, MA).

2.2.1.4 Incubated shaker

(a) Barnstead Lab Line Max Q 4000 Orbital Shaker (Fisher Scientific, Loughborough, UK).

(b) Gallenkamp Orbital Shaker Incubator.

2.2.1.5 Oven

Memmert heating cabinet (Memmert, Germany).

2.2.1.6 Plate reader

Optimax tuneable microplate reader (Molecular Devices, Sunnyvale, CA).

2.2.2 Equipment for clinical studies

2.2.2.1 Bioelectrical impedence

OMRON Healthcare (UK) Ltd, Henfield, UK.

2.2.2.2 Blood pressure and pulse measurement

OMRON Healthcare Europe BV, Hoffddorp, NL.

2.2.2.3 Filtered red light

KL 2500 LCD (Schott UK Ltd., Stafford, UK).

2.2.2.4 Plethsmograph

EC6 or EC4 plethsmography coupled with E20 rapid cuff inflator (Hokanson, WA, USA). Data displayed and analysed using Powerlab 4120 or 2120 and LabChart® Reader (Version 7.3) (AD Instruments, Oxford, UK).

2.2.2.5 Gamma counters

2.2.2.5.1 Xenon dose measurement for clinical study

Amersham radioisotope calibrator ARC-120.

2.2.2.5.2 Blood flow measurement

GMS411 Mediscint gamma counter (John Caunt Scientific Ltd, Bury, UK) supported by GMS411 Data Manager Version 1.2.0.

2.2.2.5.3 Laboratory cortisol radioimmunoassay

Wallac Wizard 1470 Gamma Counter.

2.3 Source of Materials

All reagents were obtained from Sigma (Poole, UK) unless stated otherwise.

2.3.1 Drugs for Clinical Studies

2.3.1.1 Stable isotopically labelled tracers

All stable isotopically labelled deuterated tracers were obtained from Cambridge Isotope Laboratories (CIL, Andover, MA.). Chemical and isotopic purity are as per manufacturer certificate of analysis.

2.3.1.1.1 D4-cortisol

9,11,12,12-[^{2}H]$_{4}$–cortisol powder; >98% and 98.6% isotopic enrichment as determined by gas chromatography mass spectrometry.

2.3.1.1.2 D2-cortisone

1,2-[^{2}H]$_{2}$-cortisone powder; >96.3% purity and 98% isotopic enrichment as determined by thin layer chromatography (TLC).

2.3.1.2 Unlabelled steroids

2.3.1.2.1 Hydrocortisone

Hydrocortisone sodium hemisuccinate (Solu Cortef) (Pharmacia, Belgium).

2.3.1.3 Diluents

2.3.1.3.1 Saline

Sodium Chloride (0.9% *w/v*) (Baxter, Newbury, UK), 154 mmol Sodium and 154 mmol Chloride per 1000 mL.

2.3.1.3.2 Pharmaceutical grade ethanol

Ethanol (90-100% *v/v*) Torbay manufacturing unit, Paignton, UK.

2.3.1.4 Hyperinsulinaemic euglycaemic clamp

2.3.1.4.1 Insulin

Actrapid (NovoNordisk, Crawley, UK), 100 units per mL.

2.3.1.4.2 Dextrose

Dextrose (20% *w/v*) (Baxter, Newbury, UK).

2.3.1.5 Dexamethasone tablets

Auden McKenzie (Pharma division) Ltd (a subsidiary of Actavis Ltd), Middlesex, UK.

2.3.1.6 Cortisone tablets

Martindale Pharma, Buckinghamshire, UK.

2.3.1.7 Radioactive substances

2.3.1.7.1 ^{133}Xenon

^{133}Xenon gas (USP grade; >99.5% purity) (Nordion, Ottawa, Canada). Specific activity 0.00667 mCi per μL.

2.3.2 Drugs for *in vitro* assays

2.3.2.1 Stable isotopically labelled tracers

2.3.2.1.1 D4-cortisol

9,11,12,12-[^{2}H]$_{4}$–cortisol powder (Cambridge Isotope Labotatories, Andover, MA) as above (2.3.1 Drugs for Clinical Studies).

2.3.2.1.2 D3-cortisol

9, 12,12 [^{2}H]$_{3}$-cortisol powder (Cambridge Isotope Labotatories, Andover, MA).

2.3.2.1.3 D8-cortisone

2,2,4,6,6,9,12,12 [^{2}H]$_{8}$-Cortisone Santa Cruz Biotechnology Inc (Heidelberg, Germany).

2.3.2.2 Unlabelled steroids

Cortisol, cortisone, corticosterone were obtained from Sigma (Poole, UK), epi-cortisol and epi-corticosterone were obtained from Steraloids (Newport, RI).

All of the above were made up in HPLC grade methanol at a final concentration of 1mg/mL and stored at -20 °C.

2.4 Cell Culture

2.4.1 Reagents

2.4.1.1 Fetal Calf Serum

Fetal calf serum was deactivated by heating at 60 °C for 30 minutes for laboratory use (Lonza, Slough).

2.4.1.2 Medium

Dulbecco's Modified Eagle's Medium (DMEM) (88%) containing 4.5 g/L glucose (Lonza, Slough, UK).

2.4.2 Medium Preparation

2.4.2.1 Cell growth medium

Fetal calf serum (10% *v/v*), Penicillin-Streptomycin (0.5% each *v/v*) (Gibco, Paisley, UK) and glutamine (1% *v/v*; 2mM) (Gibco, Paisley, UK) were added to DMEM.

2.4.2.2 Stripped steroid medium

As per cell growth medium but exchanging fetal calf serum with that stripped of steroid (section 2.4.4.2).

2.4.2.3 Medium for assay

As per cell growth medium except fetal calf serum omited.

2.4.2.4 Freezing medium

Fetal calf serum (50% *v/v* 25 mL) (Lonza, Slough, UK) and dimethyl sulfoxide (10% *v/v* 5 mL) (Sigma, Poole,UK) added to DMEM (40% *v/v* 20 mL).

2.4.2.5 Other reagents

2.4.2.5.1 Phosphate buffered saline

PBS (Lonza, Slough, UK).

2.4.2.5.2 Charcoal

Dextran coated charcoal.

2.4.2.5.3 Trypsin

Trypsin (200 mg/L).

2.4.2.5.4 Ethylenediaminetetraacetic acid (EDTA)

EDTA (Lonza, Slough, UK).

2.4.2.5.5 Poly-D-lysine hydrobromide

Poly-D-lysine.

2.4.3 Source of cells

Human embryonic kidney cells stably transfected with gene coding for human 11 β-hydroxysteroid dehydrogenase type I (HEK293-11βHSD1) were obtained from Drug Discovery Core, University of Edinburgh (Webster and Pallin 2007). Cells were frozen in aliquots (in 1 mL freezing medium) at -80 °C for 48 hours before being transferred to liquid nitrogen for long term storage. These were thawed rapidly upon requirement.

2.4.4 HEK293-11βHSD1 cell culture

Cells were maintained in cell culture flasks [(Corning, NY, USA); 75cm^3] pretreated with poly-D-lysine and using growth medium (section 2.4.2.1). Standard tissue

culture conditions (37 °C; 95% O_2, 5% CO_2) were used. Cells were passaged when confluent (approximately twice weekly). Cells were used at 3rd or 4th passage for assays.

2.4.4.1 Cell passage

Cell culture medium was removed from the culture plate and the cells were washed with phosphate buffered saline (5 mL). The cells were detached from the plate using trypsin (3 mL) and were shaken off after a 5 minute incubation period in standard conditions. The trypsin was deactivated using 7 mL of cell growth medium. Maximum cell recovery was ensured by rinsing the plate with medium during transfer of the cells into a 50 mL falcon tube. The cells were pelleted by centrifugation (800 x g; 22 °C) for 5 minutes and subsequently re-suspended in an appropriate volume of medium for division between flasks or wells.

2.4.4.2 Steroid removal from serum or plasma using charcoal

A suspension of dextran coated charcoal in serum or plasma (1% *w/v*) was stirred at 4 °C for 16 h. The suspension was filtered once through 3 filters (1.2, 0.45 and 0.2 μm diameter pores). The stripped serum or plasma was aliquoted and stored (-20 °C) until required.

2.4.5 Experimental conditions

HEK 293-11βHSD1 cells were trypsinised for removal from culture flasks (as per cell passage above) and re-suspended in 10 mL cell growth medium. An aliquot (200 μl) was diluted (1 in 5; total 1 mL) before being counted on a haemocytometer. One million cells were seeded into each well of a 12 well plate (Costar 12 well cell

culture plate, Corning, NY, USA). Additional cell growth medium was added (total 2 mL per well). The plates were incubated for 24 hours under standard conditions. Medium was removed and cells were washed with 1 mL phosphate buffered saline (PBS). PBS was removed and replaced with steroid stripped medium (2 mL) and incubated for 24 hours. Subsequently cells were washed in further PBS (1 mL).

2.5 Free Cortisol Assay

2.5.1 Materials

2.5.1.1 Parafilm

Parafilm (Pechiney Plastic packaging Companty, Chicago, USA).

2.5.1.2 Dialysis tubing

12-14 kDa, width 19 mm, approx length 30 m (Medicell, London, UK).

2.5.1.3 Ultrafiltration Units

Amicon Ultra-2 device (Merck, Watford, UK).

2.5.2 Reagents

2.5.2.1 Phosphate Buffered Saline

To ensure physiological pH of 7.4, phosphate buffered saline (PBS) was made in the laboratory for use in free cortisol assay. Phosphate Buffer (0.05 M) / Sodium Chloride (0.15 M) was prepared by adding KH_2PO_4 (1M, 7.6 mL), K_2HPO_4 (1M, 42.4 mL) and NaCl (5M, 30 mL) to ultrapure water (920 mL). Acidity was measured and pH adjusted by adding KH_2PO_4 (acidic) or K_2HPO_4 (basic) drop wise until pH 7.4 was achieved.

2.5.2.2 PBS gelatin

Gelatin (Hydrolysed Gelatin (Byco™ A), Croda, Goole, UK) (1% *w/v*) dissolved in PBS.

2.5.2.3 Ethylediaminetetraacetic (EDTA) acid disodium salt dihydrtate

EDTA sodium.

2.5.2.4 Ultrapure water

Milli-Q® water purification system (Millipore UK, Hertfordshire, UK).

2.5.2.5 Dialysis tubing preparation

De-ionised water containing sodium bicarbonate (2% *w/v* 10 mg) and EDTA sodium (1mM, 146.12 mg).

2.5.2.6 Dialysis tubing preservative

Ultrapure water containing 0.1% Bronidox L5 (0.1% (*v/v*), 500 μL) (Cognis, Ludwigshafen, Germany).

2.5.3 Samples and Reagents

2.5.3.1 Plasma

Plasma was obtained from clinical studies following ethical approval and informed consent. Plasma used for method development was obtained following ethical approval from the Scottish National Blood Transfusion Service, Edinburgh (Submission number 12-05).

2.5.3.2 Steroid from human plasma

Human plasma (7 mL) was stripped of steroid using dextran coated charcoal (70 mg, 1% *w/v*). The suspension was stirred at 4 °C for 16 hours before being filtered

through consecutively smaller filters (1.2, 0.45 and 0.2 μm diameter pores). Aliquots of stripped plasma (1.5 mL) were stored (-20 °C) until required.

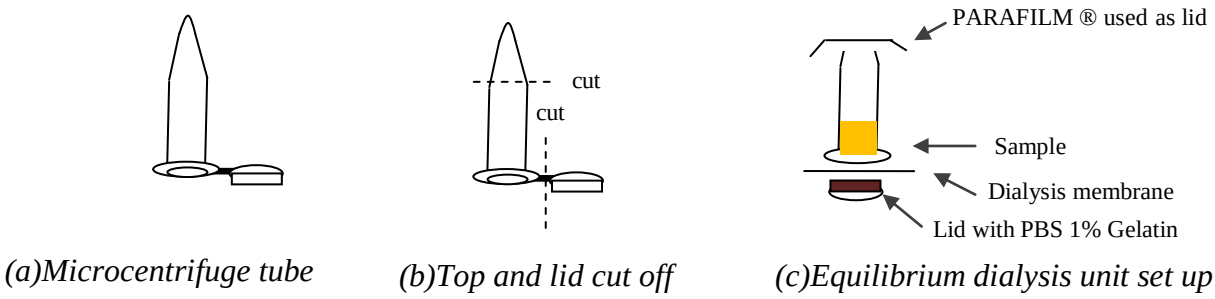
2.5.4 Equilibrium dialysis

2.5.4.1 Dialysis tubing preparation

A length of desiccated dialysis tubing was cut and heated in dialysis tubing preparation reagent (see 2.3.4.2) at 80 °C for 30 minutes. The tubing was rinsed thoroughly in de-ionised water before being stored in preservative (see 2.5.2.6).

2.5.4.2 Separation of free from bound cortisol by equilibrium dialysis

Equilibrium dialysis units were developed as a modified version of the technique instituted by Reinard et al. (Reinard and Jacobsen 1989). Lids of microdialysis tubes (Eppendorf 1.5 mL) were cut and retained. These were filled with phosphate buffered saline (250 μL) (see 2.3.4). The apex of the microdialysis tube was cut (0.5 cm from the tip). Microdialysis tubing was removed from its preservative and washed in deionised water. Strips (1.5 cm x 1.9 cm) were cut and placed over the PBS gelatin filled lid ensuring complete coverage. The dialysis membrane was held in place by rejoining the body of the tube to the lid. Plasma (1 mL) was pipetted on top of the dialysis membrane and the unit was sealed with parafilm (Figure 2.1). Equilibrium dialysis units were held securely in a plastic beaker and incubated for 16 hours (37 °C, rotated as 180 rpm). After this time the dialysate was removed from the lid and aliquoted into a glass tube for extraction (section 2.6). Where volumes of plasma greater than 1 mL were required multiple units were set up and the dialysate was pooled together.



(a)Microcentrifuge tube *(b)Top and lid cut off* *(c)Equilibrium dialysis unit set up*

Figure 2.1 Equilibrium dialysis set up

2.5.5 Ultrafiltration

2.5.5.1 Separation of free from bound cortisol by ultrafiltration

Ultrafiltration units were filled with 1 mL plasma and centrifuged at 1500 x g for 30 minutes at 37 ºC. The filtrate was collected and aliquoted.

2.5.6 Enzyme-linked immunosorbent assay

Salivary cortisol ELISA (Salimetrics, Suffolk, UK) was used since standard samples spanned a lower range of concentrations compared to alternative ELISA kits which was necessary for quantification of free cortisol.

Manufacturer's protocol was adhered to. Samples and standards (25 µL) were pipetted into the desired number of wells. Enzyme conjugate (containing antibody) was prepared by diluting stock solution 1:1600 before 200 µL aliquots were added to each well. Mixing was ensured through the use of a plate rotator (5 minutes at 500 rpm) (section 2.2.1.3) followed by a further 55 minutes at room temperature. The plate was washed 4 times with wash buffer (300 µl) provided by the manufacturer. The plate was blotted on absorbent paper to remove any residual antibody. Substrate

solution (200 µl) was added and mixed using the plate rotator (5 minutes at 500 rpm). Incubation (dark conditions at room temperature) for 25 minutes allowed maximum enzymatic conversion of the substrate. The reaction was stopped using acidic stop solution and read using a plate reader.

One well supplied by the manufacturer was used for non-specific binding and one well contained no standard or sample acting as a blank. Where detectable, non-specific binding was subtracted from all standards and samples. All samples and standards were performed in duplicate. Intra-assay relative standard deviation was accepted if < 10%.

Standards ranged 0.33 to 82.77 nmoles/L (6 standards). A 4 parameter logistic nonlinear regression curve was plotted of optical density against concentration. Coefficient of determination of the curve was accepted if $r^2 > 0.9$.

2.6 Extraction of glucocorticoids from sample matrices

2.6.1 Cell culture medium

Samples were thawed at room temperature (30 mins) before an aliquot (500 µL) was enriched with internal standard (epi-cortisol and D8 cortisone - mass used detailed in relevant section). Glucocorticoids were extracted using Sep-Pak C18 Classic Cartridges (360 mg, Waters Millipore, Watford, UK) pre-conditioned using methanol (5 mL) and water (5 mL). Sample was loaded and unwanted hydrophilic compounds were washed through with water (5 mL) before the steroid was eluted in methanol (2

mL). The organic solvent was removed by heating (60 °C) under a stream of oxygen free nitrogen (OFN). The residue was reconstituted in water (200 μL) and ethyl acetate (2 mL). The organic layer was transferred into a clean glass tube and the solvent removed by heating (60 °C) under a stream of OFN. Extracted steroids were reconstituted in mobile phase (100 μL water: acetonitrile 70:30) and transferred to LC-MS/MS vials for analysis. Where immediate analysis was not undertaken samples were stored at -20 °C.

Standards were produced for quantification by diluting stock solutions of the various glucocorticoids (1 mg/mL) into medium for assay (500 μL) ranging from 2 ng/mL to 250 ng/mL. Epi-cortisol and D8 cortisone (100 ng/mL) were used as internal standard. A blank sample containing internal standard only was included. Extraction was performed as set out above.

2.6.2 Plasma

2.6.2.1 Extraction of steroids from plasma

Plasma samples were defrosted at room temperature. Volumes (as specified in each section) were aliquoted into glass tubes. Internal standard was added (see relevant section for mass) and incorporated by vortex mixing. Chloroform was added (10:1 ratio; 10 mL chloroform: 1 mL plasma) and vortex mixed. The plasma layer was removed using a glass Pasteur pipettes and the chloroform layer transferred into a clean glass tube. This process was repeated to ensure the removal of all plasma proteins. The organic solvent was dried under OFN at 60 °C. Residual steroids were reconstituted in mobile phase (70:30 water: acetonitrile; 75 – 100 μl) before being transferred to an LC-MS/MS vial for analysis.

2.6.2.2 Preparation of standards for LC-MS/MS analysis

Stock solutions of the desired glucocorticoids (1 mg/mL) were diluted in methanol and mixed in combinations allowing for potential interference between isotopes on analysis by LC-MS/MS (separating D4-cortisol and D3-cortisol as well as cortisone and D2-cortisone). Aliquots were pipetted into glass tubes producing a concentration range as specified in each section. Internal standard identical to that used in the samples was added. The volume of each standard was made up to 200 µL with water. These were thoroughly mixed before chloroform was added (10:1 ratio; 2 mL chloroform; 200 µL standard) was added. The aqueous layer was removed and the chloroform layer was transferred into a clean glass tube. The organic solvent was removed by heating at 60 °C under OFN. The residue was reconstituted in mobile phase (70:30 H_2O: acetonitrile; volume as set out in specific section) and transferred into an LC-MS/Ms vial. If analysis was not performed immediately standards were frozen at -20 °C as per samples.

Enrichment curves were also generated representing a tracer to tracee ratio of 0 to 100% (D4 cortisol: cortisol and cortisone, D2 cortisone: cortisone and D4 cortisol: D3 cortisol). This allowed assessment of equivalent ionization of deuterated and unlabelled glucocorticoids during LC-MS/MS. Adjustment for differential ionization was only required where stated. Standards for the enrichment curve were produced using an identical method to the production of the standard curve for concentration as set out above but with consistent concentrations of cortisol and cortisone across the curve and with an incremental increase in D4-cortisol as exemplified below.

STANDARD CURVE			ENRICHMENT CURVE		
Epi-cortisol (ng)	**Cortisol (ng)**	**D4-Cortisol (ng)**	**Tracer:tracee ratio (%)**	**Cortisol (ng)**	**D4-cortisol (ng)**
500	0	0	0	500	0
500	2	1	2.5	500	25
500	5	2.5	5	500	50
500	10	5	7.5	500	75
500	20	10	12.5	500	125
500	30	15	25	500	250
500	50	25	50	500	500
500	100	50			
500	200	100			
500	300	150			
	400	200			

Table 2.1 Standard and enrichment curve

Example of a standard and enrichment curve for Cortisol and D4-cortisol quantification in plasma. In the standard curve, increasing masses of glucocorticoids are aliquoted against a fixed internal standard (epi-cortisol) from which the ratio is used to quantify unknown masses from samples. The enrichment curve provides a ratio of the mass of tracer (e.g D4-cortisol) to tracee (Cortisol).

2.7 Chromatographic Analysis

2.7.1 Instrument

LC-MS/MS:Waters Acquity™ UPLC (Manchester, UK) along with autosampler interfaced with a triple quadrupole ion trap mass spectrometer (ABSciex QTRAP® 5500 (Warrington, UK)). System operation and analyte quantification was performed using Analyst® software version 1.5.1.

2.7.2 Run conditions

Separation of deuterated and unlabelled glucocorticoids was achieved with the use of a Sunfire™ C18 column (3.5 µm, 2.1 x 100 mm) at 10 °C protected by a Hypersil C18 guard cartridge (10x3 mm, 3 µm, ThermoElectron, Hemel Hempstead, UK) . Elution with narrow distinctly separated peaks was achieved using mobile phase (0.1% formic acid in H_2O and 0.1% formic acid in acetonitrile) at a flow rate of 0.5 mL per minute. An isocratic solvent system (70:30 H_2O: acetonitrile *v/v*) was used for sample elution (6 minutes per sample).

Ionisation was performed in positive atmospheric pressure chemical ionisation mode. Mass transitions were detected using tandem mass spectrometry (curtain gas 25 psi, collision gas pressure medium, spray voltage 5kV source temperature 550 °C). Mass transitions detected are set out in Table 2.2 below.

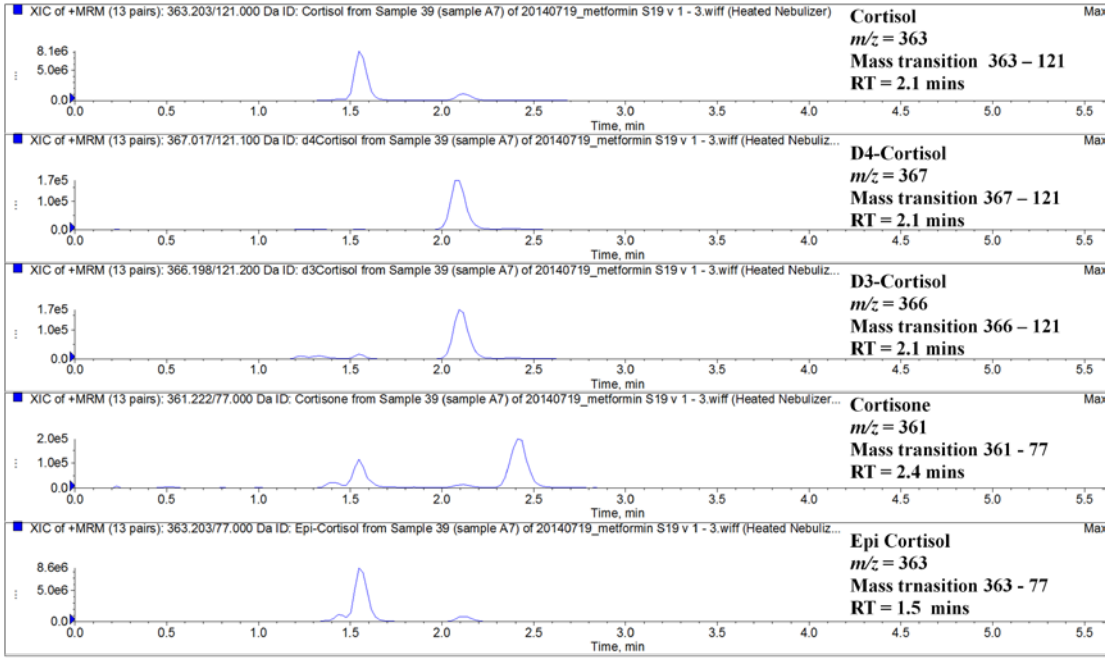


Figure 2.2 Chromatograph of deuterated and unlabelled glucocorticoids

Distinctive peak areas for each analyte separated according to mass to charge (m/z) ratio and distinctive transition for each parent ion

Analyte	*Mass transition of protonated ion (m\z)*	*De-clustering potential (V)*	*Collision energy (V)*	*Collision cell exit potential (V)*
Cortisol	363 → 121	131	19	14
Cortisone	361 → 77	166	99	36
D4-cortisol	367 → 121	121	25	20
D3-cortisol	366 → 121	121	27	20
D3-cortisone	364 → 164	166	31	14
D2-cortisone	363 → 165	166	31	22
Epi-cortisol	363 → 77	141	101	14
D8-cortisone	369 → 169	186	29	28

Table 2.2 Chromatographic conditions for LC-MS/MS

D; deuterium: *m/z*; mass to charge ratio: V; volts

2.7.3 Data analysis

Analyst® software was utilised to integrate area under the peak for each analyte and internal standard. The ratio of analyte peak area against its corresponding internal standard was used for quantification against a standard curve. A line of best fit (y = mx+c) was drawn after plotting known steroid concentrations (x axis) against the corresponding analyte to internal standard peak area ratio (y axis). A regression

coefficient (r^2) of >0.9 was accepted for each standard curve. Where D3-cortisol and D2-cortisone were quantified, separate standard curves were prepared to avoid interference between isotopologues. Enrichment curves were created to ensure corresponding ionisation between endogenous and deuterated steroids. Where this proved not to be the case steroid concentrations were adjusted accordingly as stated in the relevant section below. Arithmetic correction for background heavy isotopes was employed where necessary.

2.8 Cortisol radioimmunoassay

(Cortisol ^{125}Iodine radioimmunoassay, MP Biomedical, Hessen, Germany). All samples and reagents were brought to room temperature. Samples, standards (range 0 to 100 µg/dL) and controls were dispensed (25 µL) into individual tubes pre-coated with antibody. Competitive binding was allowed by incubating sample and radiolabelled ligand (Cortisol-^{125}I 1 mL) for 45 minutes (at 37 °C). Unbound ligand was decanted by inverting the tubes in a short sharp movement and swabbing the opening of the inverted tube with a cotton bud. Bound ^{125}I was measured using a gamma counter. All samples and standards were performed in duplicate and the mean counts per minute for each sample and standard was used for analysis. Variability was accepted if relative standard deviation was <10%. The zero standard (0 µg/dL) was used as 'total' counts from which all samples and standards were subtracted for generation of standard curve and as such determination of cortisol concentration in samples. Coefficient of determination of the standard curve was accepted if $r^2 > 0.9$.

As supplied by the manufacturer, three controls (high, medium and low) were included in each assay to assess for intra-assay variability.

Chapter 3: Effect of metformin on 11βHSD1 in obese men with and without diabetes

3.1 Introduction

Metformin has been prescribed for over 50 years and continues to be the leading treatment for type 2 diabetes mellitus (T2DM). Until recently metformin has been the only oral hypoglycaemic agent to provide favourable effects on weight (Lee and Morley 1998), lipid profile (Robinson, et al. 1998) and provide potential cardiovascular risk reduction (UKPDS (1998)). Through accumulative evidence of the deleterious effects of cortisol dysregulation, manipulation through inhibition of 11βHSD1 looked set to provide a novel pathway for treatment of both T2DM and associated metabolic syndrome features.

More than 40 programmes in pharmaceutical companies and universities have sought to develop selective inhibitors of 11βHSD1. The efficacy of such agents has proved disappointing. To date phase 2 trial data has been published for six compounds for the treatment of T2DM (Feig et al. 2011; Freude et al. 2016; Heise et al. 2014; Rosenstock et al. 2010; Shah et al. 2011) (Table 1.1) and only one has shown significant improvement in the primary endpoint of improved glycaemic control (Rosenstock et al. 2010).

As novel therapeutic agents, it is likely that 11βHSD1 inhibitors would be prescribed as second or third line agents in the treatment of diabetes thereby resulting in co-prescription with an agent such as metformin. As discussed in Section 1.11.6, the cellular mechanism of action of metformin remains unclear but more broadly likely results in reduced hepatic gluconeogenesis and inhibition of the action of glucagon.

By inhibiting intracellular generation of *de novo* glucose it is hypothesised that metformin may reduce the substrate for the co-enzyme to 11βHSD1 with resultant lowering in the availability of the co-substrate NADPH suppressing the regeneration of cortisone to cortisone in tissue. Furthermore, the co-prescription of metformin may mask any additional glucose lowering potential that 11βHSD1 inhibtitors may provide.

3.2 Hypothesis

Metformin down regulates 11βHSD1 in obese individuals with and without T2DM, but to a greater extent in the former. The focal site of action is within the liver.

3.3 Aim

1. To investigate the potential effect of metformin on 11βHSD1 activity in obese non diabetic and diabetic individuals.
2. To establish the tissue specific site of action.
3. To examine the difference in effect of metformin on 11βHSD1 between obese individuals with and without T2DM.

3.4 Methods

3.4.1 Study design

This placebo-controlled double-blinded cross over study was approved by the local research ethics committee (South East Scotland Research Ethics Committee). All subjects provided written informed consent.

3.4.2 Subjects

Five out of eight obese (BMI $\geq$ 30 kg/m^2) non diabetic (OND) participants were studied in 1996 with study visits and data collection performed by Dr G Jones. The remaining 3 participants were recruited (2012-2014) using local advertisements and the local hospital diabetes register. An identical protocol was performed for the later 3 participants as had been performed in 1996. Inclusion criteria was also consistent with the previous study and included male gender, age 18 to 70 years old, normal screening blood tests (full blood count, kidney, liver and thyroid function), no glucocorticoid use within the preceding 3 months, no other medications which may influence 11βHSD1 activity or cortisol concentrations (particularly opiates, 5-alpha reductase inhibitors, antifungal agents and regular non steroidal anti-inflammatory use except prophylactic aspirin) alcohol intake no more than recommended weekly amount and no significant change in weight (<5%) over the last 3 months. Data for the eight participants was pooled together for analysis.

For the diabetic subjects inclusion criteria specified diet or tablet controlled diabetes (on metformin monotherapy) with HbA1c less than 86 mmol/mol (<10%) if diet controlled or less than 64 mmol/mol (<8%) if on metformin. Volunteers were randomised to receive either 2 tablets metformin (500 mg each) twice daily, or 2 tablets placebo twice daily and additionally in the diabetic subjects a third phase of

two tablets gliclazide (40 mg each) twice daily. Tablets were manufactured by Tayside Pharmaceuticals with each set appearing identical. They additionally performed the randomisation ensuring equal numbers of participants started on each phase. Both investigators and participants were blinded from the treatment phase which was not decoded until completion of the study.

3.4.3 Measurements

Height, clothed weight, BMI, hip and waist circumference were measured as per standard operating procedure. A self standing height measurement scale was used to measure the participant's height to the vertex of their head with their shoes off and back of heels and head against the measuring board (to 1 decimal place). Clothed weight was measured after an overnight fast using calibrated electronic scales. Waist circumference was measured mid way between the costal margin and the iliac crest with the participant standing. The hip circumference was measured with the participant in the same position but around the maximum circumference of the buttocks. Percentage body fat and lean body mass were measured using the mean of three recordings by bioelectrical impedance again after overnight fast. Blood pressure and pulse were recorded with the volunteer sitting or lying.

3.4.4 Preparation of the stable isotope tracer (80% hydrocortisone and 20% D4-cortisol)

Hydrocortisone succinate powder (SoluCortef, Pharmacia NV/SA, Puurs, Belgium) (100 mg) was reconstituted in water for injection (50 mL) (2 mg/mL solution).

D4-cortisol was dissolved in pharmaceutical grade ethanol (1.4 mg/mL solution) and filtered in the radiopharmacy department to achieve sterility. Hydrocortisone succinate solution (1.5 mL) and deuterated cortisol solution (0.5 mL) were further diluted in 48.5 mL water for injection (total 3 mg hydrocortisone and 0.7 mg D4 cortisol in 50 mL water). This was infused as a bolus over 5 minutes (600 mL/hr).

For the continuous infusion, D4-cortisol solution (1.5 mL of 1.4 mg/mL solution) was diluted in 0.9% sodium chloride (saline) (494.3 mL) along with hydrocortisone succinate solution (4.2 mL of 2mg/mL solution) (total 8.4 mg hydrocortisone succinate and 2.1 mg D4-cortisol in 500 mL). This was infused at a rate of 82.9 mL/hour providing 1.74 mg/hr of cortisol (labelled and unlabelled; 20:80).

3.5 Clinical protocol

3.5.1 Obese non diabetic participants

The protocol for the 3 newly recruited OND participants was kept identical to that of the previously recruited 5 participants who were studied by Dr G Jones in 1996.

Visit 1: Participants were prescribed tablets for 28 days. On day 26, participants attended the Clinical Research Facility, Western General Hospital at 8am having fasted from 10pm the night before. Cannulas (20 gauge) were inserted into veins in both antecubital fossae. From one of these, baseline samples were taken for background isotopologues, fasting glucose, insulin and HbA1c. This cannula was kept patent with a slow infusion of 0.9% saline and blood samples were taken at regular intervals throughout the study visit. Through the other cannula an intravenous bolus (3.7 mg at 20% labelled cortisol enrichment) followed by continuous infusion

(1.74 mg/hr at 20% labelled cortisol enrichment) of D4-cortisol/hydrocortisone was given. Blood samples (7.5 mL tube with serum gel clotting activator) were taken at hourly intervals for the first three hours then every 15 minutes for the last hour. Serum was frozen at -40 ºC following collection.

Visit 2: The participant continued on their tablets for the following two days. Dexamethasone (0.25 mg) was ingested by the participant at 10pm on day 27 before they returned fasted at 8am the following morning (day 28). Dexamethasone was used to suppress the HPA axis allowing specific measurement of cortisol generation by 11βHSD1. One cannula (20 gauge) was inserted into the antecubital fossa. This was kept patent with a slow infusion of 0.9% saline. A baseline plasma sample was taken for measurement of cortisol and cortisone. The participant was given 25 mg oral cortisone. Blood samples were taken every 15 minutes for 150 minutes and serum separated before being stored at -40ºC.

With a half life of 6 hours (Scheen 1996), a three day washout ensured complete clearance of metformin before the participant commenced the next set of tablets and an identical protocol was followed for the subsequent visits 3 and 4.

3.5.2 Obese participants with type 2 diabetes mellitus

In order to exclude any potential influence of glycaemic control on 11βHSD1 activity, an additional phase involving gliclazide was included for diabetic subjects. To keep visits to a minimum and with the benefit of being able to measure hepatic 11βHSD1 activity on the background of isotopic tracer, study visits were reduced to one per phase which incorporated components of day 1 and 2 for the OND

participants as above. The use of steady state D4-cortisol infusion (80% unlabelled hydrocortisone and 20% D4-cortisol) will provide some HPA axis suppression hence dexamethasone was not used in the protocol in this group.

All ODM participants were on metformin monotherapy prior to commencement of the study. A three day washout period was therefore provided where no hypoglycaemic medication was taken before they were randomised to placebo, metformin and gliclazide in random order. No blood tests or intervention was performed until the study visit after taking the tablets for 28 days. Participants were asked to record capillary blood glucose readings 4 times a day for 2 days per week during each phase of the study.

On day 28 participants attended the Clinical Research Facility at 8am having fasted from 10pm the night before. Anthropometric measurements as described above as were performed on their arrival.

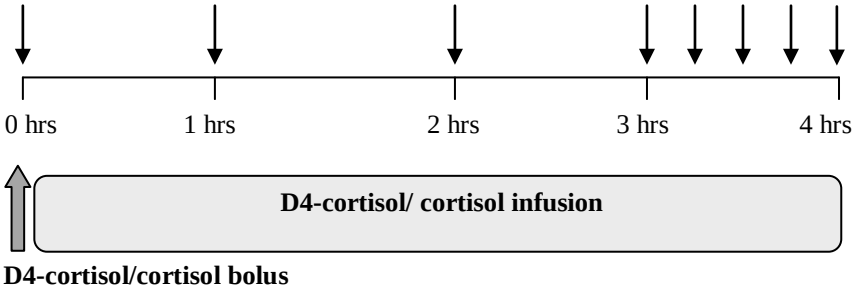
Cannulae (20 gauge) were inserted into veins in the antecubital fossae of each arm and lines kept patent with a slow infusion of 0.9% saline. Baseline samples were taken as above. A bolus and infusion of D4-cortisol/ cortisol were infused through the other cannula (as above). Blood samples were taken hourly for the first two hours then at the time points shown in the schematic below (Figure 3.1). At 180 mins, oral cortisone was administered (5mg). The dose of oral cortisone was reduced in comparison to the non diabetic subjects in view of more recent data showing that 5mg was adequate to reveal differences in hepatic 11βHSD1 activity on a background of deuterated cortisol tracer (Stimson et al. 2011). Blood samples were

obtained more frequently following ingestion up until 240 minutes. All serum samples were frozen at -40 ºC until analysis.

A three day washout period was again allowed to provide sufficient time for clearance of metformin and gliclazide (half life 11 hours) (Campbell, et al. 1991b) before commencing the next phase. The subsequent visit for the other two phases ran in an identical fashion.

OND participants

A.



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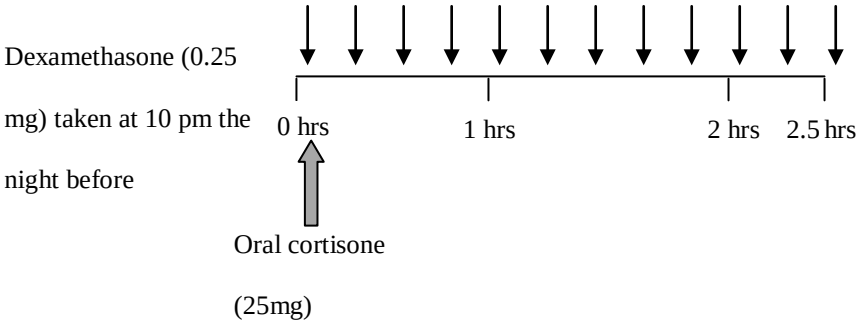
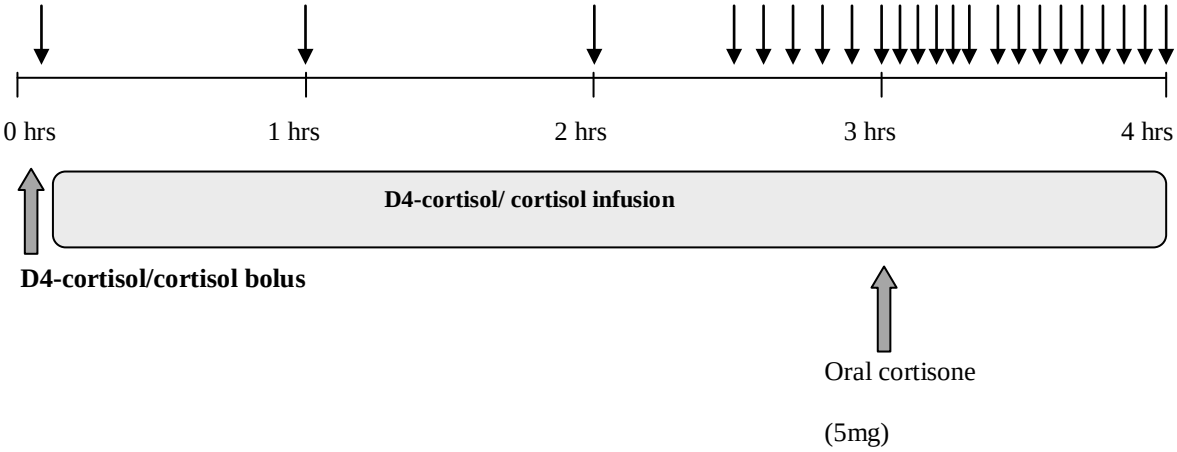


ODM participants

C.



Where; ↓= blood sample

Figure 3.1 Schematic representation of clinical study visits

Obese non diabetic (OND) (A = visit 1; B = visit 2) and obese participants with T2DM (ODM) (C)

3.6 Analytical techniques

3.6.1 Biochemistry

Measurement of biochemical indices was performed in the biochemistry laboratory, Western General Hospital, Edinburgh. Plasma glucose was measured using a calorimetric assay and insulin by immunoassay using Abbott Architect analysers. HbA1c was measured by high performance liquid chromatography (HPLC) (HA8180 analyser, Menarini Diagnostics, Berkshire, UK).

3.6.2 Whole body 11βHSD1 activity

Glucocorticoids (endogenous and deuterated were extracted from plasma (section 2.6.2.1). Samples were analysed using LC-MS/MS (section 2.7). Mass transitions for the analytes cortisol, cortisone, D4-cortisol and D3-cortisol were as laid out in table 2.2. Standard curves were prepared across the following ranges: cortisol 2 to 400ng, D4-cortisol, D3-cortisol and cortisone 1 to 200ng. Epi-cortisol (500 ng) and D8-cortisone (250 ng) were used as internal standard in all samples and standards. Standard curves were generated using the ratio of analyte peak area to internal standard peak area. For sample analysis, interference between D4- and D3-cortisol was corrected for when quantifying peak area. Analyte concentration was determined using the standard curves.

3.6.3 Hepatic 11βHSD1 activity

Cortisol concentrations following cortisone ingestion in the non diabetic individuals was measured using radioimmunoassay. As oral cortisone was administered on a background of deuterated tracer in the diabetic subjects, liquid chromatography/ mass spectrometry was used for analysis for the diabetic group. Both provide a quantification of hepatic 11βHSD 1 activity by first pass metabolism of cortisone.

3.6.4 Data Analysis

Data are presented as mean ± SEM. Data was analysed using SPSS statistics (version 21) using t-test and/or repeated measures ANOVA as indicated in the legends. Data was checked for normality of distribution using Kolmogorov-Smirnov test. Significance was taken as $p<0.05$.

3.6.4.1 Whole body data analysis

HOMA-IR was calculated as per Mathew et al.(Di Natale, et al. 2009; Matthews, et al. 1985)

Equation 1: HOMA-IR

$$\text{HOMAIR} = \frac{\text{Glucose x Insulin}}{22.5}$$

Where glucose (mmol/L) and insulin (mU/L)

Rate of appearance (Ra) of cortisol and D3-cortisol were calculated by dividing the rate of infusion of tracer by the corresponding tracer:tracee ratio (Stimson et al. 2009).

Equation 2: Ra Cortisol

$$\text{Ra Cortisol} = \left(\frac{Rate\ of\ D4{-}cortisol\ infusion}{D4{-}cortisol/cortisol}\right) - \ rate\ of\ cortisol\ infusion$$

Equation 3: Ra D3-cortisol

$$\text{Ra D3-cortisol} = \left(\frac{Rate\ of\ D4{-}cortisol\ infusion}{D4{-}cortisol/D3{-}cortisol}\right)$$

Cortisol and D4-cortisol clearance (L/min) were calculated by dividing their rate of infusion by their steady state concentration.

3.6.4.2 Hepatic data analysis

In the non diabetic subjects, the activation of cortisone to cortisol was calculated using area under the curve by plotting cortisol concentrations against time. For the diabetic subjects who had concurrent administration of intravenous tracer, Steele's non steady state equation was applied (Equation 4).

Equation 4: Steele's non steady state equation

$$\text{Ra cortisol (t)} = \left(\frac{\text{infusion rate of D4 cortisol}}{E(t)}\right) - \left(\frac{V \times \frac{C(t)}{1+E(t)} \times \frac{dE(t)}{dt}}{E(t)}\right)$$

Where t denotes time, V is volume of distribution, C(t) is total cortisol concentration at time (t) and E(t) is the tracer to tracee ratio (D4 cortisol/cortisol).

Since the volume of distribution for cortisol is unknown this was taken as being 12 L as has been done for calculation of cortisol generation in additional studies (Andrew et al. 2005; Dube, et al. 2014; Dube et al. 2015b).

3.7 Results

One subject from the OND group had to be removed from the first phase of the study due to gastrointestinal side effects from the tablets. No data was collected from this individual whose data have therefore not been included in the results below. One of the non diabetic participants reduced the dose during the metformin phase to one

tablet once daily due to the development of diarrhoea. Removal of this subject's data had no significant impact on analysis and has therefore been included in kinetic calculations.

3.7.1 Participant characteristics

Subjects with T2DM (n = 8) were significantly older than the non diabetic individuals (mean ± SD: OND participants 43.6 ± 13.0 vs ODM participants 65.8 ± 2.3; $p < 0.05$). HbA1c was statistically greater in individuals with T2DM. BMI was similar between the two groups (Table 3.1). All ODM participants were on statin therapy which was continued throughout the study.

	Obese non diabetic participant (n=8)	Obese participants with type 2 diabetes mellitus (n=8)
Age (years)	43.6 ± 13.0	65.8 ± 2.3*
BMI (kg/m^2)	37.4 ± 7.2	34.7 ± 3.1
HbA1c (mmol/mol)	40.1 ± 5.4	48.2 ± 4.1*

Table 3.1 Baseline characteristics of non diabetic and diabetic participants

Data = mean ± standard deviation. *=$p<0.05$ unpaired t-test

3.7.2 Biochemical data

No ODM participants experienced hypoglycaemia. A trend was observed for variation in fasting glucose between phases in ODM participants (Table 3.2). As anticipated, neither metformin nor gliclazide treatment resulted in a reduction in HbA1c. After 28 days, metformin did not result in a significant reduction in insulin resistance, as assessed by HOMAIR, in either ODM or OND. Only one participant in the ODM group had significant weight loss (4%) with metformin treatment. Weight, body mass index and fat mass remained steady in all other participants in both groups throughout the study.

	OND		ODM		
	Placebo	**Metformin**	**Placebo**	**Metformin**	**Gliclazide**
Fasting glucose (mmol/L)	5.6 ± 0.6	5.3 ± 0.2	9.6 ± 1.0	7.4 ± 0.6*	8.5 ± 0.8
HbA1c (mmol/mol)			55.4 ± 2.5	52.4 ± 3.0	53.6 ± 3.6
Insulin (mU/L)	17.1 ± 5.1	10.6 ± 2.2	24.2 ± 9.7	24.9 ± 10.3	21.4 ± 7.5
HOMA-IR	5.0 ± 1.9	2.5 ± 0.6	9.4 ± 3.2	7.0 ± 2.2	7.2 ± 1.7
Cholesterol (nmol/L)	4.5 ± 0.4	4.7 ± 0.6	3.5 ± 0.2	3.4 ± 0.2	3.4 ± 0.2

Table 3.2 Biochemical effects of metformin and gliclazide in OND and ODM participants

Analysis using repeated measures ANOVA * $p < 0.1$ Placebo vs metformin vs gliclazide; $p = 0.02$ placebo vs metformin; $p= 0.15$ placebo vs gliclazide; $p = 0.14$ metformin vs gliclazide

3.7.3 Rates of appearance of cortisol and D3-cortisol measured by tracer kinetic

3.7.3.1 Non diabetic tracer data

On the background of steady state D4-cortisol infusion, the dilution with both cortisol and D3-cortisol was greater following metformin treatment (Figure 3.2). Statistical comparisons were undertaken using average results during steady state (180-240 mins infusion). The rate of production of D3-cortisol was not normally distributed and data was transformed using $\log_{10}$ for statistical analysis.

Clearance of both cortisol and D4-cortisol was not significantly different between the two phases. Rate of production of cortisol was not significantly different between groups. Metformin treatment did, however, significantly increase the generation of D3-cortisol (Table 3.3).

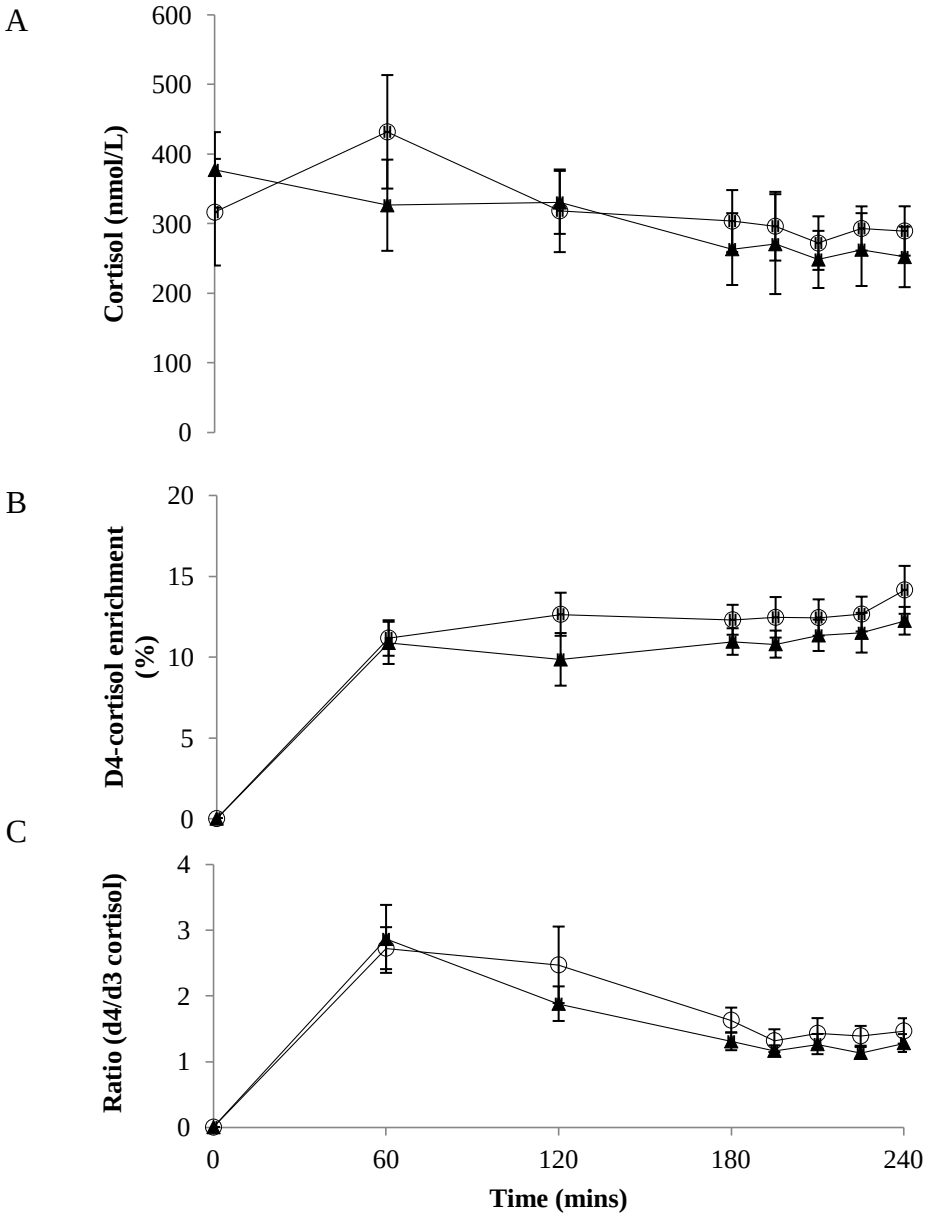


Figure 3.2 The effect of metformin treatment in OND individuals

Data are mean ± SEM for plasma measurements taken over a 4 hour infusion of deuterated cortisol (D4-cortisol). Placebo (o; open circles); metformin (▲; triangles). Cortisol concentrations (nmol/L) (Graph A); Enrichment of D4-cortisol with cortisol (%) (Graph B); D4 cortisol: D3-cortisol tracer to tracee ratio (TTR) (Graph C)

3.7.3.2 Diabetic patient tracer data

Steady state was taken at the earlier time point of 150-180 minutes (Andrew, et al. 2002). Metformin did not alter the clearance of cortisol or D4-cortisol. No significant difference was observed in the rate of appearance of endogenous cortisol but metformin did result in a significant increase in the generation of D3-cortisol compared with placebo and gliclazide treatment (Table 3.3). On a background infusion of D4-cortisol, the generation of D3-cortisol is specific to 11β-reductase activity. This result therefore implicates an increase in reductase activity by 11βHSD1 with metformin treatment.

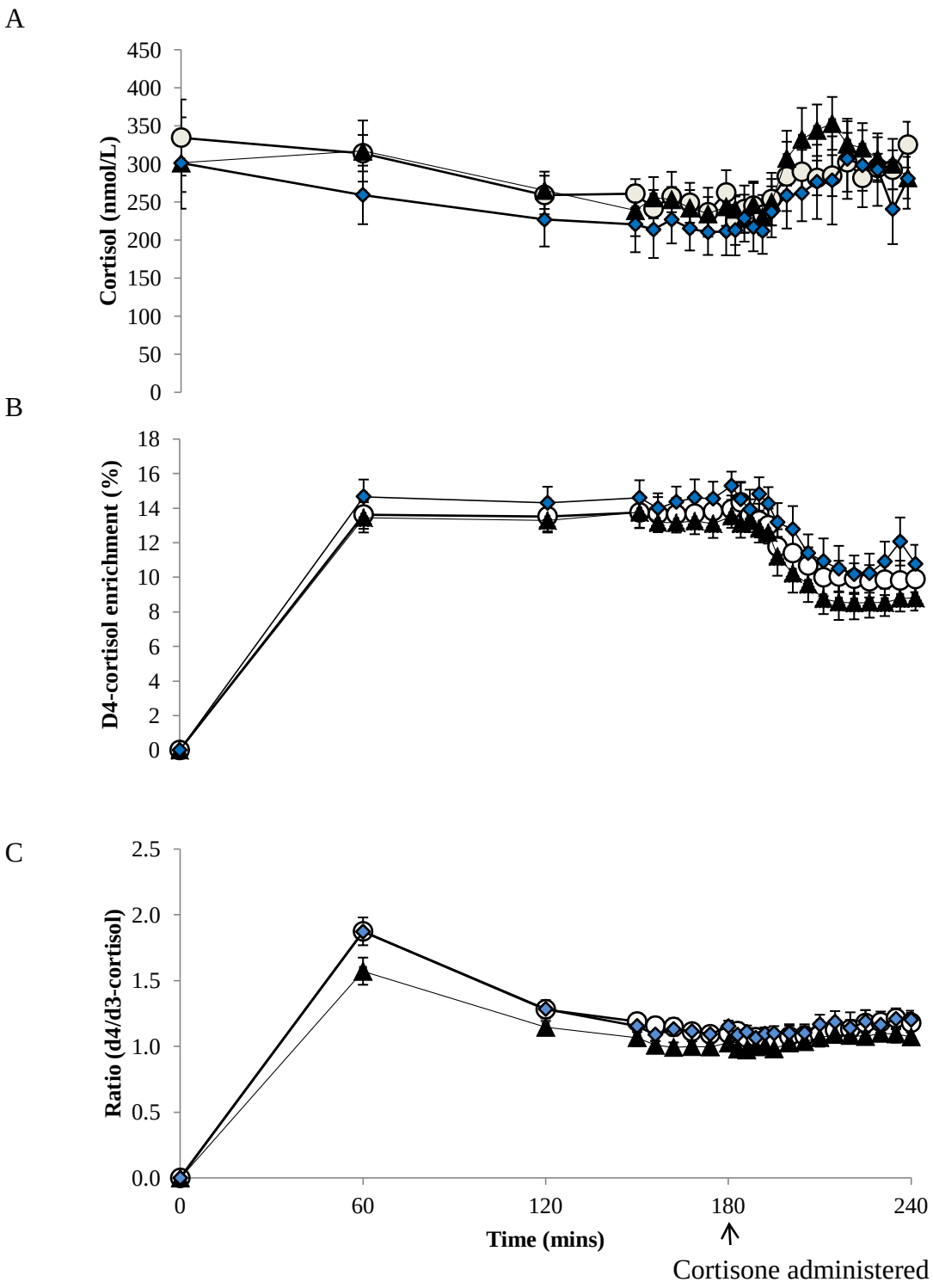


Figure 3.3 The effects of metformin and gliclazide treatment in ODM participants

Data are mean ± SEM for measurements taken over a 4 hour infusion of D4-cortisol. Placebo (o; open circles); metformin (▲; black triangles); gliclazide (◆ ; blue diamond). Cortisol concentrations (nmol/L) (Graph A); Enrichment of D4-cortisol with cortisol (%) (Graph B); D4-cortisol: D3- cortisol tracer to tracee ratio (TTR) (Graph C)

	OND		ODM		
	Placebo	**Metformin**	**Placebo**	**Metformin**	**Gliclazide**
Cortisol clearance (L/min)	0.23 ± 0.02	0.28 ± 0.04	0.27 ± 0.03	0.28 ± 0.03	0.34 ± 0.05
D4-cortisol clearance (L/min)	0.46 ± 0.02	0.61 ± 0.1	0.51 ± 0.06	0.53 ± 0.06	0.58 ± 0.08
Ra cortisol (nmol/min)	70.4 ± 17.6	79.7 ± 15.0	54.4 ± 7.75	57.89 ± 6.71	48.2 ± 8.38
Ra D3-cortisol (nmol/min)	11.2 ± 1.4	12.9 ± 1.2*	14.21 ± 0.63	15.78 ± 0.58$^{\#}$	14.21 ± 0.54

Table 3.3 Steady state tracer kinetics data OND and ODM participants

Ra; Rate of appearance
OND: Analysis using student's t-test *= $p<0.05$ metformin vs placebo
ODM: Analysis using repeated measures ANOVA $^{\#}$ = $p<0.01$; $p<0.01$ placebo vs metformin; $p = 0.99$: placebo versus gliclazide; $p = 0.02$ metformin vs gliclazide

3.7.4 Hepatic 11βHSD1 activity

Using area under the curve, hepatic 11βHSD1 activity was calculated after oral cortisone ingestion for non diabetic subjects. Metformin treatment did not significantly alter the conversion rate of cortisone to cortisol (placebo 121,659.6 ± 23,171.3 vs metformin 111,109.9 ± 1,7418.1 nmol/L x min).

In the ODM group, Ra cortisol was calculated using Steele's non steady state equation (Equation 4). No significant difference was seen for either rate of appearance of cortisol (placebo 89.11 ± 10.35 vs metformin 106.50 ± 11.67 vs gliclazide 81.28 ± 11.43 nmol/min; p=0.45) or area under the curve under the curve (placebo 5329.40 ± 651.19 vs metformin 6416.21 ± 685.15 vs gliclazide 4438.92 ± 749.78 nmol/L/min; p=0.3) (Figure 3.4). By analysing the maximum rate of production a trend was observed for metformin to increase the rate of production of cortisol compared to gliclazide (maximum rate of production: placebo 127.2 ±23.5 vs metformin 163.4 ± 19.6 vs gliclazide 106.5 ± 15.5 nmol/min; p <0.1).

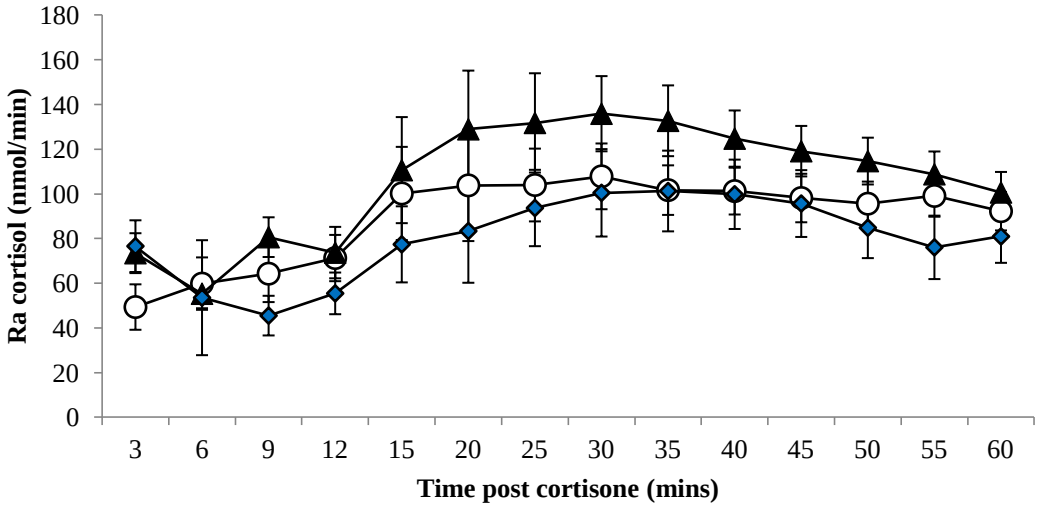


Figure 3.4 Rate of appearance of cortisol following oral cortisone administration in ODM group

Data are mean ± SEM at intervals post oral cortisone ingestion (5mg). Placebo (o; open circles); metformin (▲; black triangles); gliclazide (◆; blue diamond)

3.7.5 Obese non diabetic versus diabetic participants

Using steady state D3-cortisol production rate (without cortisone administration), a two way ANOVA was conducted to compare whole body 11βHSD1 activity between OND and ODM individuals and the impact of metformin treatment. The rate of appearance of D3-cortisol was significantly greater in ODM compared with OND individuals. No significant interaction was observed with metformin treatment (Figure 3.5).

The rate of appearance of cortisol was not significantly different between obese individuals with metformin treatment with or without T2DM (Two way repeated measures ANOVA OND placebo 70.44 ± 17.63 vs OND metformin 79.73 ± 15.01 vs ODM placebo 54.42 ± 7.75 vs ODM metformin 57.89 ± 6.71 nmol/min; p = 0.21 OND vs ODM; p = 0.84 OND vs ODM vs metformin treatment).

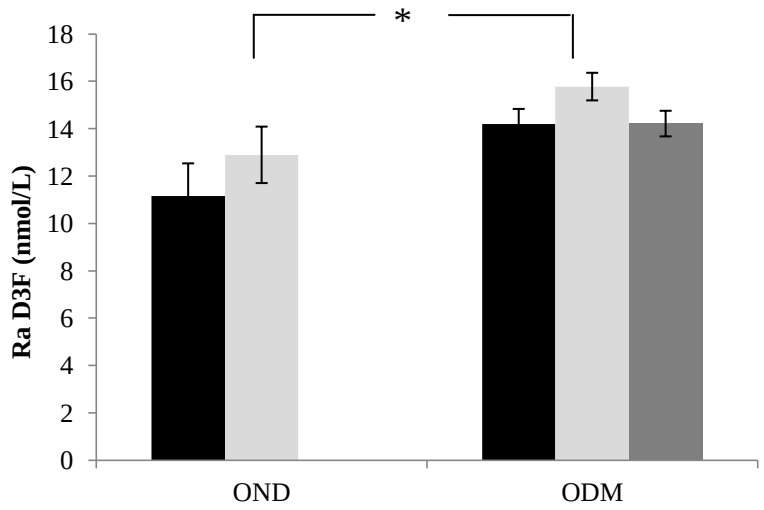


Figure 3.5 Rate of appearance of D3-cortisol in OND and ODM participants

OND Obese non diabetic individuals; ODM obese individuals with T2DM. Black bar: placebo; light grey bar metformin; dark grey bar: gliclazide treatment.
Two way repeated measures ANOVA * p = 0.018 OND vs ODM; p = 0.765 OND vs ODM vs metformin treatment

3.8 Discussion

This chapter reports the investigation of the effect of metformin treatment on the regulation of cortisol through the action of 11βHSD1.

For the first time we have observed increased whole body 11βHSD1 activity in obese men with T2DM compared with euglycaemic counterparts. No significant difference was observed between the two groups in rate of production of endogenous cortisol. A study comparing healthy lean individuals, obese euglycaemic and obese individuals with T2DM revealed a non significant increase in cortisol production between obese

non diabetic and obese diabetic individuals but no difference was observed in 11βHSD1 activity between the groups (Basu et al. 2005). This could be explained by higher circulating insulin concentrations in our participants compared with the study by Basu et al. resulting in greater availability of co-substrate.

A study performed by Stimson et al. showed upregulated whole body 11βHSD1 activity in obese diabetic patients compared with lean non diabetic individuals (Stimson et al. 2011). From this it was proposed that in euglycaemic obesity hepatic 11βHSD1 activity is down regulated counterbalancing increased activity in alternative tissue such as adipose. With the addition of relative insulin deficiency as in T2DM, relatively increased hepatic 11βHSD causes further dysregulation resulting in elevated whole body cortisol regeneration. The results from this study would be in keeping with this hypothesis.

Contrary to our hypothesis, we have additionally shown that metformin treatment upregulates whole body 11βHSD1 activity in obese individuals both with and without T2DM. This effect was independent of the glucose lowering action of metformin as shown through the lack of effect from an additional hypoglycaemic agent, namely gliclazide. Metformin has long been thought to improve insulin sensitivity in T2DM (Section 1.11.6) although evidence for this is conflicting (Hällsten, et al. 2002; Hundal, et al. 1992; Hundal and Inzucchi 2003; Sirtori and Pasik 1994). With improved insulin sensitivity cellular glucose may increase with metformin treatment providing increased rather than the hypothesised decrease in co-substrate for 11 βHSD1. Alternatively, metformin and perhaps AMPK may have a direct effect on 11βHSD1 transcription increasing cellular regeneration of cortisol

with an aim of benefiting from the catabolic effects in restoring the balance of ATP/AMP.

Five out of the eight OND individuals were studied a number of years ago but it is reassuring that the effect of metformin on 11βHSD1 in these individuals is reflected similarly in the more recent data.

In the knowledge that hepatic 11βHSD1 activity is sustained in individuals with T2DM (Stimson et al. 2011) and as the major site of extra adrenal cortisol production (Andrew et al. 2005; Stimson et al. 2009; Stimson, et al. 2007) it was hypothesised that the liver would underlie any elevation in whole body 11βHSD1 activity secondary to metformin treatment. This was suggested by the data but not confirmed. As a secondary aim, the study was not powered to detect difference in hepatic 11βHSD1 activity and further studies would therefore be required to investigate this further.

Dube et al. recently developed a triple tracer technique to measure hepatic 11βHSD1 activity without the need for invasive procedures (Dube et al. 2014). No difference was observed in hepatic 11βHSD1 reductase activity between lean non diabetic, obese non diabetic individuals or obese diabetic individuals. The BMI of the obese group varied widely between 27 and 40 kg/m^2.

In this study we utilised the principle of first pass metabolism for the conversion of cortisone to cortisol by hepatic 11βHSD1. In the OND group we calculated area under the curve for the generation of cortisol whilst in the ODM group, with background D4-cortisol tracer infusion, we used Steele's non steady equation as has

been performed in other similar studies (Stimson et al. 2011). With administration of oral cortisone and subsequent conversion to cortisol, the ratio of D4-cortisol to cortisol is altered disrupting the steady state balance (Stewart et al. 1999). As a result of a relative reduction in deuterated substrate, whole body 11βHSD1 activity appears to decrease. This can be overcome by titrating the infusion of D4-cortisol to compensate keeping kinetics in steady state. More recently Dube et al. have developed a triple tracer technique using oral [4-^{13}C]cortisone for the measurement of reductase activity of 11βHSD1 and [9,12,12-$^{2}H_3$]cortisol (deuterated cortisol) for the measurement of cortisol extraction across liver. This is performed on a background infusion of (1,2,6,7-^{3}H)cortisol (tritiated cortisol) allowing measurement of hepatic 11β-reductase activity using isotope dilution technique.

The increase in 11βHSD1 activity may alternatively originate from adipose tissue. The use of *ex-vivo* adipose tissue biopsies would help investigate this further. Metformin does not appear to up-regulate alternative glucocorticoid metabolising enzymes as shown by the lack of significant increase in clearance of cortisol or deuterated cortisol. Measuring excreted urine metabolites in urine would additionally prove this.

With the additional control phase with gliclazide treatment in the ODM participants, the protocol was altered to allow measurement of whole body and tissue specific activity of 11βHSD1 in one visit thereby reducing the total number of study visits. The set-up for measurement of whole body enzyme activity was identical between the two groups allowing a comparison to be made. This is not the case for the measurement of hepatic 11βHSD1 activity. For the ODM participants a smaller dose

of cortisone was administered which may have masked any increase in hepatic 11βHSD1 activity with metformin treatment. In fact a trend was observed in this group that was not seen using the higher dose of cortisone in the OND group. Performing this test early in the morning, as was done with the OND group, has the potential to be influenced by higher circadian cortisol synthesis by the adrenal gland producing a false positive result. This was accounted for by administering dexamethasone to suppress the HPA axis. This was not performed in the ODM group although they had background tracer (80:20 unlabelled hydrocortisone: D4-cortisol mixture) at steady state before the administration of cortisone which may provide some adrenal suppression and additionally benefits from allowing any increased metabolism of cortisol by other enzymes, such as the 5α and 5β-reductase enzymes, to be accounted for. Overall without arteriovenous sampling and measurement of blood flow across the liver, the measurement of hepatic 11βHSD1 activity in this study is more qualitative than quantitative hence the impact of differences between protocols is less important with the biggest disadvantage being the inability to compare the effect of metformin on hepatic 11βHSD1 activity between the two groups.

3.9 Conclusion

In conclusion, we have shown that metformin upregulates whole body 11βHSD1 activity in obese diabetic and non-diabetic individuals. The site of action is likely to be the liver but increased participant numbers and *in vitro* studies would be required to elucidate a mechanism of action. Contradictory to our hypothesis, co-prescription

of metformin may enhance rather than attenuate the efficacy of 11βHSD1 inhibitors. The results of studies to date looking at 11βHSD1 inhibitors as novel treatment for T2DM have not been masked by co-prescription with metformin.

Chapter 4: Free cortisol

4.1 Introduction

11βHSD1 is located within the endoplasmic reticulum. Cortisol must therefore first dissociate from its carrier protein and pass through the cell membrane before being metabolised by 11βHSD1. The findings from clinical studies of tissue specific dysregulation of 11βHSD1 in subcutaneous adipose of obese individuals (Paulmyer-Lacroix, et al. 2002; Wake, et al. 2003) and in liver of both obese non diabetic (Rask et al. 2001; Rask et al. 2002) and diabetic individuals (Stimson et al. 2011) have relied on total plasma cortisol measurements, that is the measure of free plus bound concentrations of circulating cortisol. This assumes rapid equilibration between free and bound hormone and equal binding affinity and capacity of cortisol to CBG between subjects.

There are several reasons why this may not hold true. Inflammation and more specifically the effect of neutrophil elastase is perhaps the most widely recognised influence affecting cortisol binding. With infection and inflammation neutrophil elastase is released and cleaves CBG dissociating cortisol from its binding sites. This results in an overall disproportionately higher free cortisol concentration (Ho, et al. 2006). In such situations total cortisol levels can be misleading in the assessment of glucocorticoid status (Hamrahian, et al. 2004) and measurement of free cortisol may be a better prognostic marker in conditions such as sepsis (Fede, et al. 2014; Ho et al. 2006).

Cortisol binding is altered in the setting of more chronic inflammation as occurs with the metabolic syndrome. From a cohort in Spain, free cortisol concentrations correlated significantly with blood pressure, waist to hip ratio and HOMA-IR

(Fernandez-Real et al. 2002). As well as the effect of neutrophil elastase, the binding site on CBG can become glycosylated causing a conformational change and as a result lowering binding affinity for cortisol (Chan et al. 2013). Sex and age have additionally been observed to influence circulating free cortisol (Purnell, et al. 2004).

It is therefore evident that using total cortisol levels may not convey the magnitude of 11βHSD1 activity and perhaps free cortisol measurements would be more representative.

No gold standard method exists for the measurement of free cortisol. Mathematical calculations have been formulated but rely on assumptions of binding protein availability and affinity. The two best recognised means of separating free from total cortisol are ultrafiltration and equilibrium dialysis. These have been compared with varying results (Pretorius, et al. 2011; Vogeser, et al. 2007). The choice of technique may be dependent on inescapable factors such as cost and time, and efficiency is often dependent upon the skill set of the operator. The small magnitude of free cortisol concentrations requires sensitive tools for detection and quantification. With wider availability this is now most commonly provided by LC-MS/MS (Kirchhoff, et al. 2011).

4.2 Hypothesis

Measurement of 11βHSD1 turnover in the total pool underestimates 11βHSD1 activity.

4.3 Aim

(a) To develop a reproducible method for the measurement of free cortisol.

(b) To compare the rate of turnover between cortisone and cortisol by 11βHSD1 assessed using the total and free pool.

4.4 Method development

Ultrafiltration and equilibrium dialysis were compared for sensitivity and reproducibility for the measurement of free cortisol concentrations in plasma. Plasma was stripped of steroid (section 2.5.3.2). Cortisol was added in increasing concentrations (125, 250 and 450 nmoles/L) and incubated (4 hours at 37°C). Ultrafiltration and equilibrium dialysis were performed (section 2.5.4 & 2.5.5) using 1 mL plasma for ultrafiltration and 250 μL for equilibrium dialysis. Alteration in sample volume (concentration with ultrafiltration and dilution with equilibrium dialysis) were adjusted for calculating free cortisol concentration as outlined below.

For equilibrium dialysis plasma (500 mL) was dialysed against PBS gelatin (250 μL) (Section 2.5.4). With free passage of unbound cortisol across the dialysis membrane and incubation in preset conditions (37 °C rotated at 180 rpm for 16 hours) free

cortisol will distribute equally between each volumetric portion i.e. each 250 µL portion. Measurement of cortisol mass in the 250 µL dialysate (PBS Gelatin) was multiplied by the dilution factor.

Equation 5: Adjustment of free cortisol mass for dilution by equilibrium dialysis

$$Cortisol\ mass\ (ng)\ \ x\left(\frac{sample\ volume\ (\mu L) + dialysate\ volume(\mu L)}{dialysate\ volume\ (\mu L)}\right)$$

i.e. for dialysis of 250 µL plasma against 250 µL PBS gelatin =

$$Cortisol\ mass\ \ x\frac{(250 + 250)}{250}$$

And for dialysis of 1000 µL plasma against 250 µL PBS gelatin =

$$Cortisol\ mass\ \ x\frac{(1000 + 250)}{250}$$

The process of ultrafiltration results in filtration of all unbound cortisol through a membrane into a volume of filtered plasma matrix. Preliminary lab work revealed that this volume was variable following ultrafiltration of plasma (1mL) (Data are mean ± SEM filtrate volume; 150.22 ± 10.64 µL; relative standard deviation 17.35%) providing a mean concentration of volume of 6.66 times.

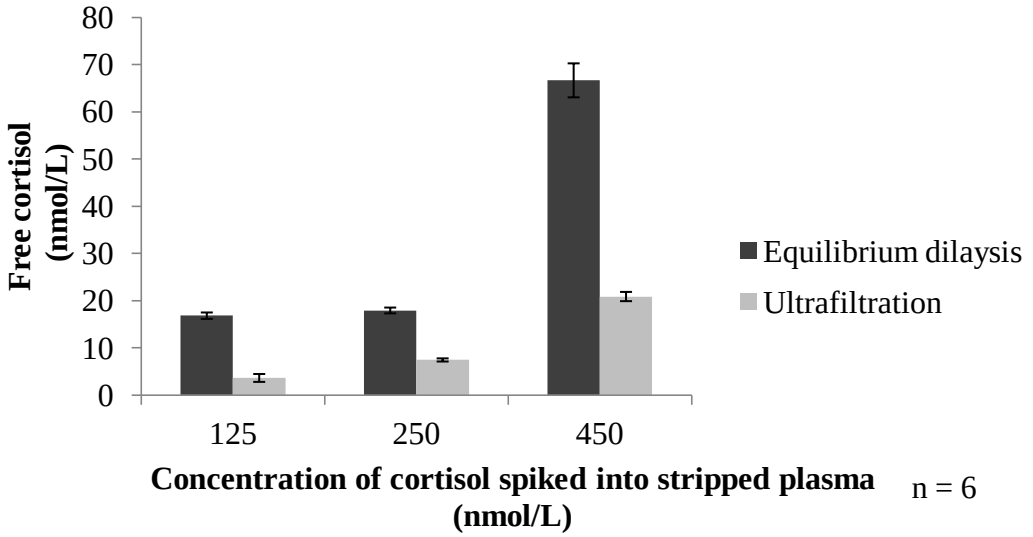


Figure 4.1 Free cortisol concentration using equilibrium dialysis and ultrafiltration across a range of total cortisol concentrations

Free cortisol rose disproportionately as measured using equilibrium dialysis when cortisol spiked into plasma reached concentration 400 nmol/L postulated to be the result of exceeding binding capacity of CBG (section 1.4).

	Intra-assay relative standard deviation (%)		*Inter-assay relative standard deviation (%)*	
Cortisol spiked into stripped serum (nM)	*Ultrafiltration*	*Equilibrium dialysis*	*Ultrafiltration*	*Equilibrium dialysis*
125	51.3	9.9	19.6	6.9
250	10.9	8.4	12.8	6.7
450	9.4	10.8	12.0	5.7

Table 4.1 Intra and inter-assay variability across a range of concentrations

Intra-assay n = 6; inter-assay n = 4
Relative standard deviation = (standard deviation / mean) x 100

4.4.1 Discussion

No gold standard method exists for the separation of free from bound cortisol. Equilibrium dialysis and ultrafiltration are the best studied in literature although neither appears superior to the other. The choice between these two methods in published studies appears to be based on cost, simplicity of assay and time involved rather than on measures of accuracy and precision (Barre, et al. 1985; Pretorius et al. 2011; Vogeser, et al. 2002).

Our data has shown that equilibrium dialysis resulted in least variability as assessed by coefficient of variation both intra and inter-assay. No gold standard method currently exists for measurement of free cortisol in plasma to compare accuracy of the two methods. It is noted that equilibrium dialysis consistently produced higher free cortisol concentrations than ultrafiltration as has been seen in published data (Vogeser et al. 2007). It is recognised that volume dilution alters protein-ligand equilibrium in a sample which may explain this (Geiseler and Ritter 1984). The above experiments used a ratio of plasma sample to filtrate of 2 to 1. The equilibrium dialysis units have a maximum capacity of 1000 μL sample against a fixed dialysate volume of 250 μL. Utilizing this sample to dialysate volume ratio of 4 to 1 may therefore cause least disruption of the existing equilibrium between free and bound cortisol when separating out the free cortisol pool.

With reduced variability both inter and intra-assay, equilibrium dialysis was utilized in further experiments following optimization of sample volume.

4.4.2 Optimisation of sample volume for equilibriation dialysis

The optimum sample volume dialysed against a fixed volume of dialysate (250μL PBS gelatin) was investigated. Plasma obtained from the Scottish Blood Transfusion Service was dialysed in increasing volumes (250, 500, 750 and 1000 μL) against 250 μL PBS Gelatin. A 4:1 plasma to dialysate ratio displayed lowest variability between samples (Figure 4.1). Higher free cortisol concentrations were observed with a sample to dialysate ratio of 1 to 1 postulated to be the result of disruption of the equilibrium between free and bound ligand.

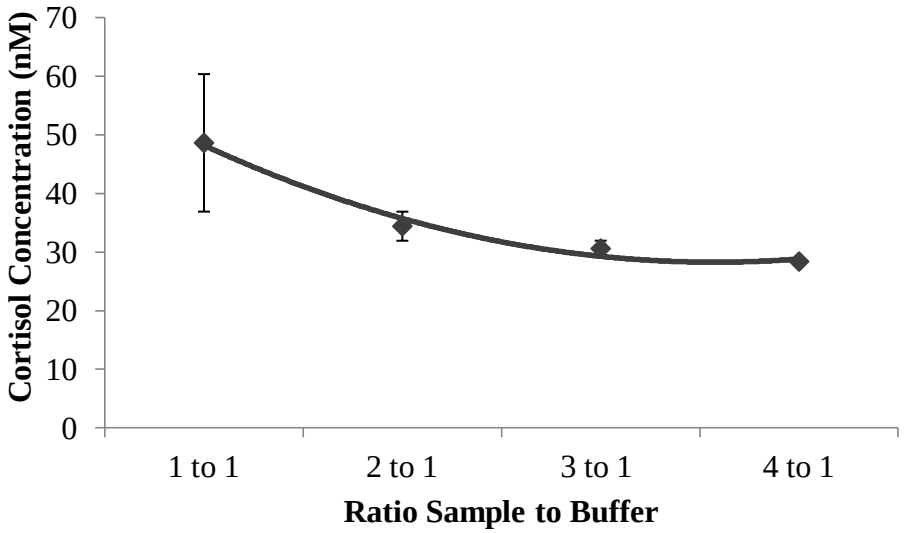


Figure 4.1 Variation in free cortisol concentration in response to increasing plasma sample volume

Data are mean ± SEM n= 4

4.4.2.1 Use with cortisol tracer

Before using this method for the analysis of plasma samples from clinical studies, the reproducibility for the measurement of free deuterated cortisol was tested. The same protocol was therefore performed using stripped plasma (section 2.4.4.2) spiked with D4-cortisol. Plasma was incubated with D4-cortisol (450 nM for 4 hours at 37°C). Plasma (1000 μL) was then dialysed against PBS gelatine (250 μL) for 16 hours. The dialysate was extracted (section 2.6) before analysis using LC-MS/MS (section 2.7). Reproducibility was acceptable with an intra-assay coefficient of variation 2.6% (SEM 0.08, n = 6). Mean percentage free cortisol (free/total x 100) approximated that expected for normal physiology (mean ± SEM; 6.2% ± 0.07).

4.4.3 Limit of detection and limit of quantification

The sensitivity of LC:MS/MS for measurement of free deuterated cortisol was investigated.

Stripped plasma was spiked with increasing concentrations of cortisol and D4-cortisol. Samples (1 mL) were dialysed and extracted as above. Samples were run using the LCMS method previously described (section 2.7).

The limit of detection was quantified as the lowest concentration to produce a mean signal to noise ratio of greater than or equal to three. The limit of quantification was taken as the lowest concentration which was reproducible with a coefficient of variation of less than 20% (Armbruster and Pry 2008).

The limit of detection for cortisol, D4-cortisol and D3-cortisol was 0.2 ng on column with a limit of quantification for the same of 0.5 ng on column. This meant that a 9 mL plasma sample would be required to accurately measure cortisol and its isotopes under conditions previously utilized for clinical *in vivo* studies.

4.5 Cortisol turnover in the free pool

A study undertaken within our department by Stimson et al. (Stimson et al. 2014) showed acute perturbation in 11βHSD1 activity through altered diet composition. Whether this alteration would be reflected through assessment of the free pool was unknown.

In summary the study enrolled eight healthy normal weight men aged 18 to 70 years who were randomised in a single blinded cross over study. Deuterated cortisol

(0.35 mg per hour following a bolus of 0.7 mg) was infused for 180 minutes achieving steady state in plasma. After 210 minutes, participants received an isocaloric liquid meal composed of carbohydrate, protein or fat or a low calorie placebo drink. Plasma samples were taken for measurement of cortisol, D4 cortisol and its metabolites. From this 11βHSD1 activity was calculated as has been described in section 3.6.

4.5.1 Original study results

Steady state total cortisol concentrations in all participants and across all diets were similar. The high protein meal led to greater endogenous cortisol production whereas a high carbohydrate meal increased whole body 11βHSD1 activity, as shown by an increased rate of production of D3-cortisol. A high fat meal had some effect on increasing endogenous cortisol production but had no significant effect on 11βHSD1 activity.

4.5.2 Methods

To satisfy the limits of quantification, a 9 mL sample was required. Due to residual sample availability, samples had to be pooled together (Table 4.2). This provided 4 pre steady state samples, 8 steady state samples and 3 subjects with samples across 3 diets from placebo, high carbohydrate diet and high protein diet (9 samples). Pre steady state samples were included to investigate whether equilibrium of D4- and D3-cortisol occurred at an earlier time point in the free compared with the total pool.

	Time	*No. samples*
Pre steady state	60 – 120 mins	4 samples *(8 subjects pooled together in duplicate)*
Steady state	180 - 210 mins	8 samples *(8 individual subjects)*
Isocaloric meal	210 – 390 mins	9 samples *3 subjects with samples across 3 meals* *(carbohydrate, protein and placebo)*

Table 4.2 Plasma samples pooled together from clinical study

Plasma was vortex mixed before being dialysed (1 mL aliquot dialysed against 250 µL PBS Gelatin) (section 2.5.4). Dialysates were drawn together in a glass tube before being mixed and extracted (as per section 2.6.2.1).

Rather than comparing the results of free isotopolouges to original published data which was analysed using a different platform, a non dialysed sample of plasma (1 mL) was simultaneously extracted and analysed alongside.

Analytes were reconsitituted in mobile phase (100 μL) before being analysed using LC-MS/MS (section 2.7). Standard curves were generated to quantify cortisol, D4-cortisol and D3-cortisol (range 0.25 to 20 ng) using internal standard (epi-cortisol 6.25 ng).

The rate of appearance of cortisol and D3-cortisol were calculated as described in section 3.6 and as had been performed in the original study.

4.5.3 Results

Pre steady state samples showed that equilibrium had been reached (time t+ 60-120 minutes) between the free and total pool as shown by very similar rates of production of cortisol and D3-cortisol (Ra cortisol free pool vs total pool; 70.0 ± 12.9 vs 66.9 ± 12.5 nmol/min: Ra D3-cortisol free pool vs total pool; 14.3 ± 0.5 vs 14.3 ± 0.5 nmol/min respectively) (Figure 4.2). During steady state there was no difference in rate of production of cortisol or D3-cortisol as assessed using the free and total pools (Ra cortisol free pool vs total pool, 59.0 ± 7.2 vs 54.9 ± 6.6 nmol/min; Ra D3-cortisol free pool vs total pool, 20.1 ± 1.1 vs 19.4 ± 1.0 nmol/min respectively).

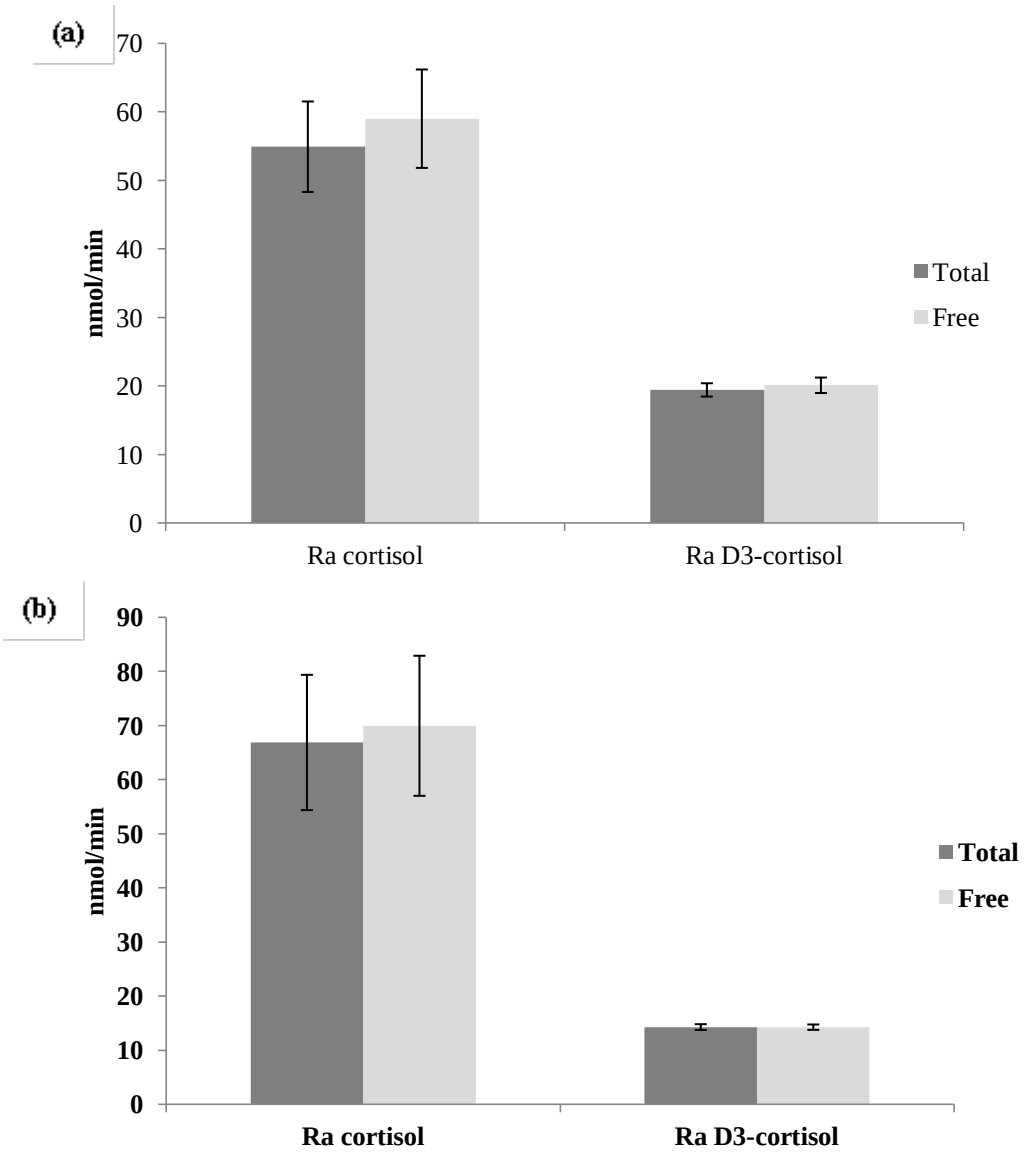


Figure 4.2 Rate of appearance of cortisol and D3-cortisol in free and total plasma pool

(a) pre steady state (60 – 120 mins) and (b) during steady state (time t+ 180 – 210 mins). Total cortisol: dark grey: Free cortisol: light grey. Mean data ± SEM. n = 4

4.5.3.1 Turnover within the free pool

As can be seen across the time course below acute perturbation of both endogenous cortisol and D3-cortisol was reflected to a similar degree in the free compared with the total pool (Figure 4.3). With the small sample size (n = 3) in each group, kinetic calculations and statistical analysis was not undertaken.

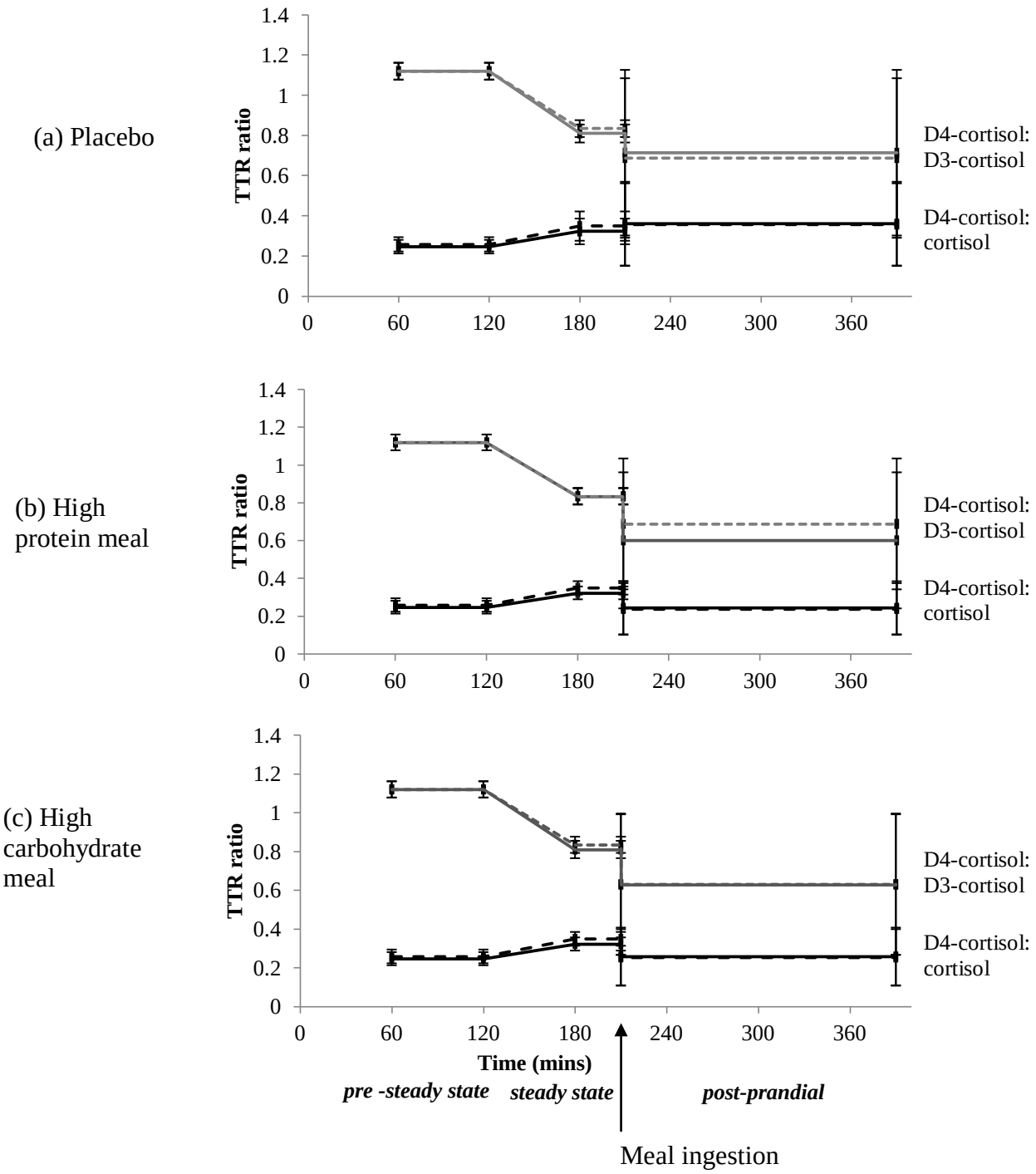


Figure 4.3 Tracer to tracee ratios in free and total pool

(a) Placebo (b) High protein and (c) High carbohydrate meal.
Tracer to tracee ratio (TTR) D4-cortisol: cortisol: total pool; dashed black line: free pool; solid black line
Tracer to tracee ratio D4-cortisol: D3-cortisol: total pool; dashed grey line: free pool; solid grey line

4.5.4 Discussion

In this chapter we have developed a method for separating free from bound cortisol and used this to compare turnover by 11βHSD1 in the free compared with total plasma pool. Using samples from a previous clinical study we have shown that turnover between cortisol and cortisone is reflected similarly in the total compared with the free plasma pool. Clinical studies to date have therefore not underestimated 11βHSD1 activity.

We observed greater precision with equilibrum dialysis compared with ultrafiltration. As discussed above comparative studies have found little difference in free cortisol measurements between these two techniques and decision of superiority has been based on practical considerations such as cost, ease and time of assay. Equilibrium dialysis has been used in clinical studies to measure free cortisol in the setting of sepsis (Ho et al. 2006), alterations across gestation in pregnancy (Clerico, et al. 1980), and in the assessment of the HPA axis (Vogeser et al. 2002). With no gold standard method and little data on normal reference ranges it is difficult to evaluate accuracy of each method.

Both techniques lead to unavoidable alteration in volume from the initial sample resulting in the need for an additional corrective calculation when working out the final concentration. Ultrafiltration results in a concentration of sample volume whilst equilibrium dialysis leads to dilution. The use of a small molecule with a low binding affinity could be used as an internal standard eliminating the need for volume measurement and adjustment. Radiolabelled glucose (14C glucose) has previously been utilised (Adachi, et al. 1991; Hammond, et al. 1980).

The samples used in this study were collected 3 years previously and had been stored at -80°C since then. Endogenous glucocorticoids are stable when stored in freezing conditions and show little variability in concentrations with repeated freezing and thawing (Kley and Rick 1984). The rate of appearance of cortisol and D3-cortisol at steady state in the total pool in this study was compared with those from the original study. The Ra D3-cortisol, which utilises the ratio of D4-cortisol to D3-cortisol, was very similar (mean ± SEM; original study 15.6 ± 1.4 nmol/min vs this study 19.4 ± 1.0 nmol/min) whilst the Ra cortisol, which utilises the ratio D4-cortisol to cortisol showed a greater discrepancy between the original and this study (original study 36.1 ± 7.3 nmol/min vs this study 54.9 ± 6.6 nmol/min). This provides additional reassurance of the stability of deuterated cortisol in freezing conditions.

The binding and dissociation of cortisol to circulating proteins is a dynamic process which *in vivo* establishes equilibrium. It is possible that at sites of inflammation where maximal cortisol is required dissociation from CBG occurs to a greater extent than in the body as a whole. As discussed in Chapter 1, binding affinity of cortisol to CBG is altered with changes in pH, increased temperature and proteases. Plasma samples used in this study were from healthy volunteers reducing the potential impact of circulating inflammatory cytokines on CBG binding affinity. Samples were maintained at 37 °C during equilibrium dialysis reducing disruption of ligand to protein equilibrium.

Through the use of plasma samples from pre-steady state tracer infusion, we have shown that cortisol equilibrates rapidly between the free and bound plasma pool. This was further confirmed with similar tracer to tracee ratios following acute

perturbation. Due to the low concentration of free deuterated cortisol and the sensitivity of LC-MS/MS, large plasma volumes were required. This meant multiple samples from different time points were required to be pooled together.

It would be of interest to compare the rate of appearance of cortisol and D3-cortisol in the free and total pool in obesity associated with a low-grade inflammatory state.

4.6 Conclusion

In conclusion through the use of equilibrium dialysis and measurement of turnover between cortisone and cortisol by 11βHSD1 in the free pool, clinical studies to date have not underestimated whole body 11βHSD1 activity.

Chapter 5: Cortisol-cortisone recycling by 11βHSD1

5.1 Introduction

Early studies showing an inverse relationship between measures of central adiposity and fasting cortisol concentrations recognized altered glucocorticoid metabolism with increasing central obesity (Marin et al. 1992). With isolation and characterization of the cortisol regenerating enzyme 11βHSD1 (Agarwal, et al. 1989), *in vivo* studies began to dissect tissue specific dysregulation in relation to obesity and the metabolic syndrome.

In obesity increased cortisol regeneration by 11βHSD1 has been observed in adipose tissue (Rask et al. 2001; Rask et al. 2002; Sandeep et al. 2005; Stimson et al. 2009). Hepatic 11βHSD1 activity is correspondingly down-regulated with euglycaemic obesity but is sustained in the presence of T2DM postulated to be the result of insulin deficiency (Stimson et al. 2011). The latter finding has not been consistent (Basu et al. 2005; Dube et al. 2015a).

The effects of insulin on whole body and tissue 11βHSD1 activity have been variable. A study comparing the effects of hyperinsulinaemia between lean and obese individuals showed acute downregulation of adipose 11βHSD1 activity in lean but not obese individuals postulated to be the result of altered co-enzyme and co-substrate (Sandeep et al. 2005). In healthy male individuals hyperinsulinaemia resulted in upregulated 11βHSD1 reductase activity in whole body and adipose tissue (Wake et al. 2006). No *in vivo* data exists for the direct effect of insulin on hepatocytes but can be inferred in individuals with T2DM (Stimson et al. 2011) and with alteration in diet composition (Stimson et al. 2014). Hepatocytes cultured with high insulin concentrations display a reduction in 11βHSD1 mRNA but with little

effect on enzyme activity as measured in the reductase direction (Jamieson et al. 1995). Different tissues may respond differently to hyperinsulinaemia and evidence exists from *in vivo* studies that the effect of insulin may relate to acute versus chronic changes (Stimson et al. 2007; Stimson et al. 2014).

A main hypothesis has been that 11βHSD1 acts exclusively as a reductase enzyme *in vivo* and as such assessment of 11βHSD1 activity and interpretation of altered mRNA and protein levels has focused on the consequences of local cortisol regeneration from cortisone. However, a recent study undertaken within the department using D2-cortisone tracer to measure dehydrogenase activity as well as the D4-cortisol tracer to measure reductase activity has shown bidirectional activity by 11βHSD1 *in vivo* with a ratio of reductase to dehydrogenase activity of 1:1.3 in adipose tissue and 1:1 in skeletal muscle (Hughes et al. 2012). If confirmed, this suggests that variations in 11βHSD1 expression and alterations in 11 β-reductase activity, may not have the predicted effect on intracellular cortisol levels since there may be simultaneous up-regulation of 11 β-dehydrogenase activity. This could explain the disappointing effects of some 11βHSD1 inhibitors on glycaemic control in T2DM, if there were inhibition of both dehydrogenase and reductase activities. Moreover, since reductase activity can be abolished in favour of persisting dehydrogenase activity by the removal of H6PDH (Lavery et al. 2006) it may be that variations in cofactor availability control the balance between dehydrogenase and reductase activities of 11βHSD1 *in vivo* in humans and this mediates the context-dependent effects of insulin on enzyme activity.

5.2 Hypothesis

11βHSD1 acts as a bidirectional enzyme catalyzing reductase and dehydrogenase reactions with directionality in favour of reductase activity in obese individuals which is further enhanced with hyperinsulinaemia

5.3 Aims

1. Confirm bidirectional activity of 11βHSD1in intact cells using deuterated tracer *in vitro*
2. Quantify directional activity using deuterated cortisol and cortisone tracers *in vitro*
3. Quantify directional activity of 11βHSD1 in whole body and across tissue in lean and obese individuals using deuterated cortisol and cortisone tracers.
4. Assess the effect of altered cellular environment with hyperinsulinaemia on directional activity of 11βHSD1 in whole body and across tissue in lean and obese individuals using deuterated cortisol and cortisone tracers.

5.4 Directionality of 11βHSD1 *in vitro*

5.4.1 Methods

HEK-293 cells stably transfected with 11βHSD1 (HEK 293-11βHSD1) (Section 2.4.3) were thawed rapidly and cultured in cell growth medium (section 2.4.2.1) in culture flasks pre-treated with Poly-D-lysine under standard tissue culture conditions (section 2.4.4). Cells were split every third day once confluent. For experimental use cells ($1x10^6$) were seeded onto 12 well plates pre-coated with poly-D-lysine and

incubated for 24 hours in growth medium. PBS was used to wash the cells before replacing the medium for stripped steroid medium (section 2.4.2.2) for 24 hours.

Stock solutions of deuterated cortisol (D4 cortisol) (1 mg/mL) and unlabelled cortisone (1 mg/mL) were diluted in serum free medium (medium for assay 2.4.2.3) to achieve a concentration of 500 nmol/L. After thorough mixing, 1 mL of medium with either D4-cortisol or cortisone was aliquoted into the desired number of wells allowing experiments to be performed in triplicate. Simultaneous control experiments were performed with the omission of cells. At pre-determined time points (6, 12, 24 and 48 hours) the medium was harvested and transferred into a glass bijou. Samples were frozen at -20 °C until analysis was performed.

Glucocorticoid extraction from the medium was performed and standards generated as described previously (section 2.6.1). Standards and samples were analysed by LC-MS/MS (section 2.7). Standard curves were generated (section 2.6.1) from which concentrations of deuterated and endogenous glucocorticoids in the samples could be calculated.

5.4.2 Results

Analysis of control samples using LC-MS/MS found detectable naturally occurring isotopologues of cortisol with mass to charge ratio equivalent to that of D4- and D3-cortisol. The mean of these was subtracted from the mean mass of the experimental samples at each time point. Data are reported as mean ± SEM.

When incubated with cortisone, HEK 293-11βHSD1 cells generated cortisol reaching maximum mass by 24 hours. Cortisone concentrations fell and leveled out correspondingly.

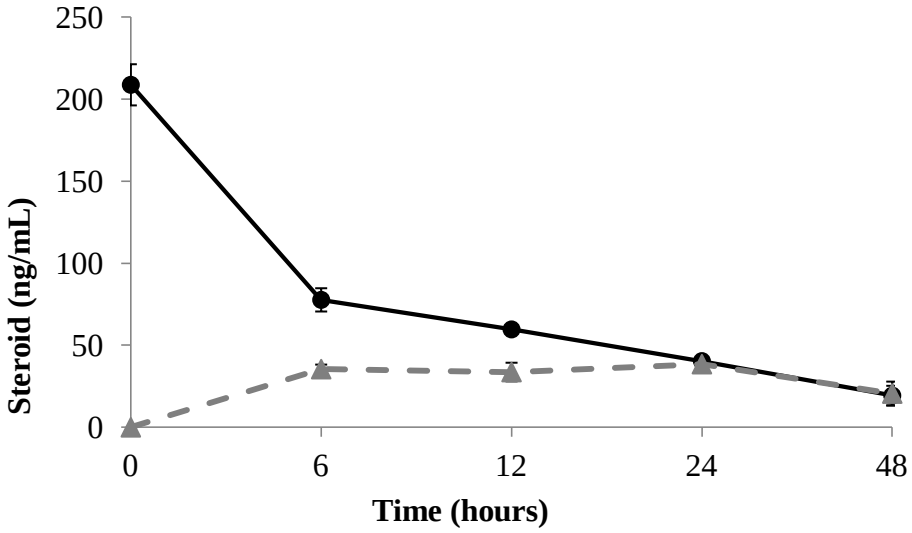


Figure 5.1 Conversion of cortisone to cortisol by 11βHSD1 in HEK293-11βHSD1 cells

Black circles: cortisone; Grey triangle: Cortisol. Mean data ± SEM. n=9

Incubation of HEK 293-11βHSD1 cells with D4-cortisol generated D3-cortisone after 6 hours. D4-cortisol mass showed little variation in the first 6 hours despite this rapid increase in generation of D3-cortisol. With a natural abundance of 0.01%, the potential for spontaneous generation of D4-cortisol or D3-cortisol is negligible. It is therefore possible that the measured mass of D4-cortisol at time point 0 was erroneous. The concentration of D4-cortsiol in the sample time+ 0 mins lay at the

top of the standard curve range (range 2 ng/ml to 250 ng/ml (Section 2.6.1)) which may explain this lower than anticipated result.

This increase in D3-cortisol was not did not correspond Generation of D3-cortisol peaked by 12 hours and remained steady for the subsequent 36 hours. Production of D3-cortisol rose steadily over the entire 48 hours. D4-cortisol mass declined between 12 and 48 hours.

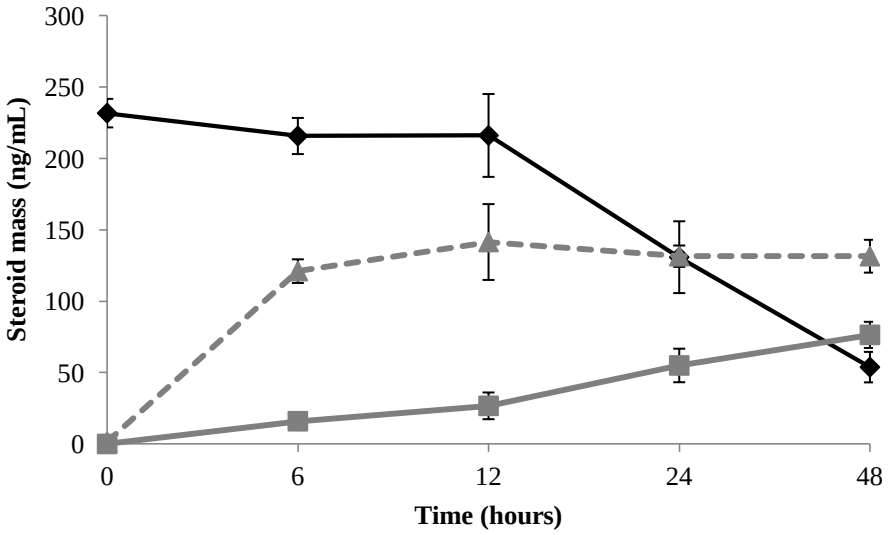


Figure 5.2 Dehydrogenase and reductase activity by 11βHSD1 following incubation of HEK293-11βHSD1 cells with D4-cortisol

Black diamonds: D4-cortisol; grey triangle: D3-cortisone; grey square: D3-cortisol. Mean data ± SEM. n=9

5.4.3 Discussion

Our work has shown that 11βHSD1 can act both as a reductase and dehydrogenase enzyme as previously observed *in vitro* (Lakshmi and Monder 1988) and *in vivo* (Hughes et al. 2012). Having been stably transfected with 11βHSD1, HEK293-11βHSD1 cells must catalyse the dehydrogenation of D4-cortisol to D3-cortisone

generation before adequate substrate is available for reduction to D3-cortisol. As such this appeared to result in a lag time before the regeneration of cortisol in the form of D3-cortisol.

Application of results from *in vitro* studies to human physiology can be limited due to the false fixed environment in which *in vitro* studies are performed. It is therefore of interest how alteration in cellular metabolism may affect enzyme directionality and overall cellular balance between active and inactive glucocorticoids.

5.5 Cortisol-cortisone recycling *in vivo*

5.5.1 Methods

5.5.1.1 Study design

This random-order two-phase crossover single-blinded study was approved by the local research ethics committee (South East Scotland Research Ethics Committee). All subjects provided written informed consent.

5.5.1.2 Subjects

Ten lean (BMI 20-25kg/m^2) and ten obese (BMI >30kg/m^2) healthy male volunteers were recruited. Each participant attended for a screening visit to ensure inclusion criteria were met (age between 20 to 70 years, no significant systemic illness or history of diabetes mellitus, no glucocorticoid use in the preceding 3 months, alcohol intake less than 21 units per week, normal screening bloods (full blood count, random glucose, kidney, liver and thyroid function) and no regular anticoagulation use).

5.5.1.3 Measurements

Participants attended the Clinical Research Facility, Royal Infirmary Edinburgh at 8am having fasted from 10pm the night before. Measurements were taken of clothed height, weight, waist and hip circumference as well as percentage body fat (as per section 3.4.3).

5.5.1.4 Preparation of stable isotope tracer

5.5.1.4.1 D4-cortisol

Deuterated cortisol (D4-cortisol) was diluted in pharmaceutical grade ethanol (90%) (1.4 mg/mL) and filtered to achieve sterility. Further dilution (1.5 mL of 1.4 mg/mL solution) in 0.9% saline (19.5 mL) was undertaken before a bolus (total dose 1.4 mg) was infused over 5 minutes. After this time a continuous infusion (3.4 mL of 1.4 mg/mL stock solution in 325 mL 0.9% saline) was infused over the study period of 5 hours (total dose 3.56 mg).

5.5.1.4.2 D2-cortisone

Stock solution was made by diluting deuterated cortisone (D2-cortisone) in pharmaceutical grade ethanol (90%) (230 μg/mL). For the bolus, 0.4 mL was diluted in 18.4 mL 0.9% saline (total dose 76 μg) and infused over 5 minutes. For the continuous infusion 2.4 mL of 230 μg/mL stock solution was diluted in 247.6 mL 0.9% saline. Work within our department has shown that primed infusion of D2-cortisone rapidly reaches steady state (Hughes et al. 2012) hence this was commenced at time+ 60 minutes. Total dose infused over 4 hours was 421.21 μg.

5.5.1.4.3 Preparation of insulin infusion

Actrapid (0.3 mL of 100 units/mL) was diluted in 0.9% saline (100 mL) creating a concentration of 0.3 units/mL. Rate of infusion was 35 mU/m^2/h based on surface area as calculated below (Du and Du 1916).

Equation 6: Calculation of body surface area

Body surface area (m^2) = $\sqrt{\frac{weight\ x height}{3600}}$

5.5.2 Clinical protocol

A cannula (20-gauge) was inserted into a vein in the antecubital fossa. This was used for infusion of dueterated cortisol (D4-cortisol) and cortisone (D2 cortisone). Three further retrograde cannulas were inserted to measure 11βHSD1 reductase and dehydrogenase activity across whole body, forearm muscle and abdominal subcutaneous adipose tissue (Figure 5.3).

One cannula (20-gauge) was inserted into a vein on the dorsum of the hand which was placed in a heated box (controlled at 60°C; manufactured in house) for 5 minutes prior to sampling in order to arterialize the blood (oxygen saturation confirmed as > 98%). This provides a measure of glucocorticoid concentrations in whole body and entering tissue without the need for more invasive arterial cannulation. This technique has been used in several other studies (Dube et al. 2015b; Stimson et al. 2009; Stimson et al. 2011). The second (20-gauge) was placed in a branch of the cubital vein near the antecubital fosse on the opposite side. Blood gas measurement ensured oxygen saturation < 40%. An inflatable cuff was placed at the wrist and

inflated to 200 mmHg for 2 minutes prior to sampling to minimize contamination of venous blood from the hand. The third cannula (18-guage) was placed in a branch of the superficial epigastric vein in subcutaneous adipose tissue of the abdominal wall. This was inserted under guidance of a filtered red light (section 2.2.2). The tip of this cannula was kept above the inguinal ligament to prevent contamination from venous drainage from the leg. Oxygen saturation was confirmed as being > 85%. These cannulas were kept patent with a slow infusion of 0.9% saline. Infusion was stopped and dead space withdrawn before blood samples were obtained. Samples were taken at the time points as outlined in below.

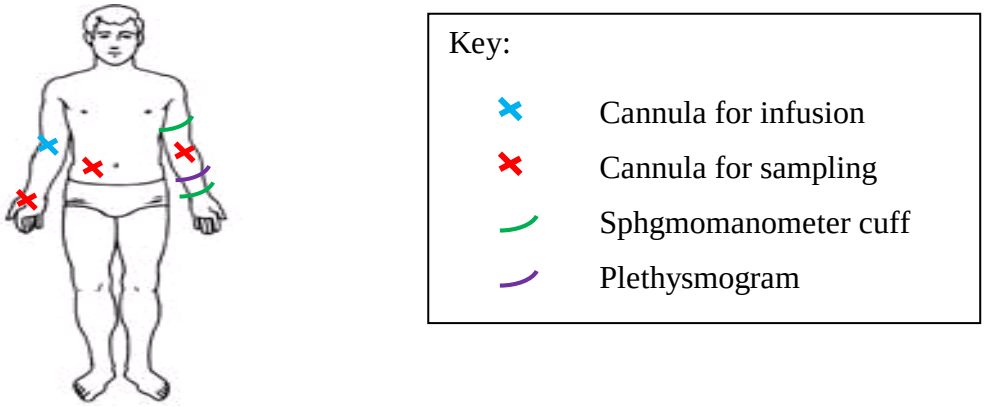


Figure 5.3 Study set-up

5.5.2.1 Blood flow measurement

At sampling time points blood flow was measured through forearm muscle and abdominal subcutaneous adipose tissue. For forearm muscle blood flow venous occlusion plethsymography was utilized (section 2.2.2.4) (Benjamin, et al. 1995). A strain gauge was applied across the mid forearm and blood flow obstructed at the wrist using a cuff inflated to 200 mmHg. Intermittent obstruction then release of

venous outflow above the forearm creates dilation of the arm which is detected by the strain gauge and corresponds to arterial blood flow rate.

Xenon^{133} (section 2.3.1.7.1) was used for blood flow measurement through abdominal subcutaneous adipose tissue (Larsen, et al. 1966). Following dilution, a dose of 2MBq was drawn up (section 2.2.2.5.1) and injected lateral to the umbilicus. A gamma counter was secured in place at the site of injection and was set to measure activity every 20 seconds throughout the study (section 2.2.2.5.2).

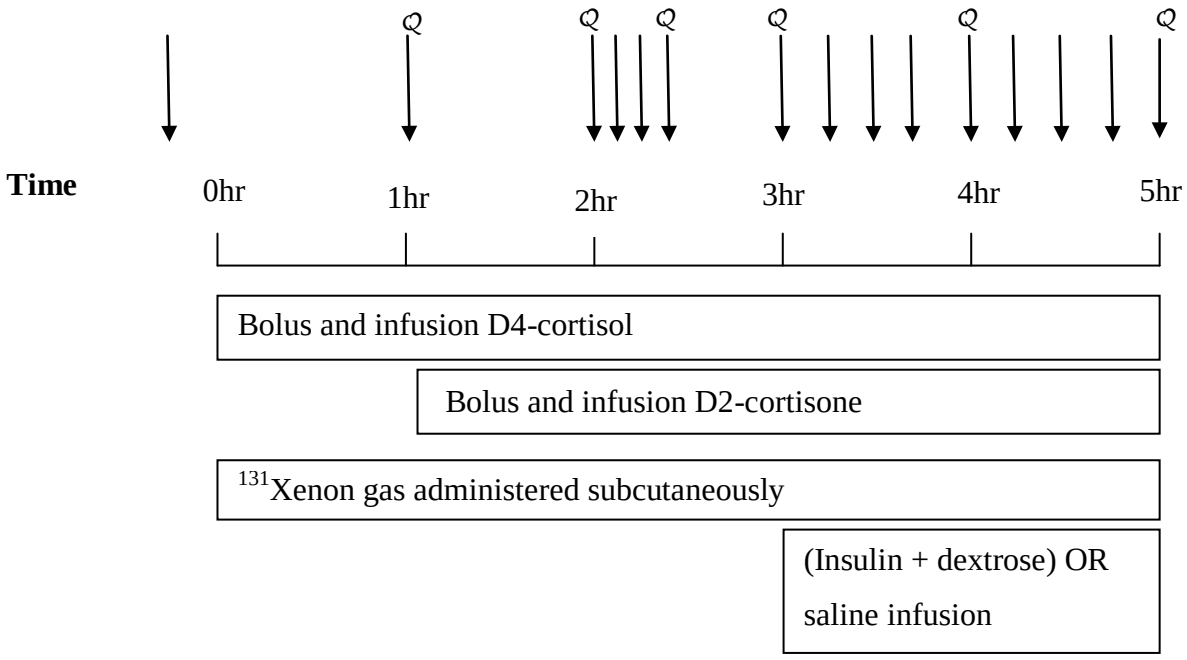


Figure 5.4 Schematic of protocol

↓ = sampling point and Q = blood flow measurement

5.5.2.2 Hyperinsulinaemic euglycaemic clamp

At one visit subjects received a hyperinsulinaemic euglycaemic clamp and at the other an infusion of saline as placebo. Patients were randomized (performed by research nurse at Clinical Research Facility, Royal Infirmary, Edinburgh) and blinded to which they were receiving. Capillary glucose was recorded every 5 minutes for the duration of the infusion (insulin or placebo). A dextrose infusion (10%) was run alongside the insulin infusion and the rate adjusted to maintain capillary glucose between 4.5 and 5.5 mmol/L.

5.5.3 Analytical techniques

5.5.3.1 Whole body and tissue specific 11βHSD1 activity

Glucocorticoids (endogenous and deuterated) were extracted from plasma (section 2.6.2.1). Samples were analysed using LC-MS/MS (section 2.7). Mass transitions of the analytes were as per Table 2.2. Standard curves were prepared across the following ranges: cortisol 2 to 400 ng; D4-cortisol 1 to 200ng; D3-cortisol 1 to 200 ng and D2-cortisone 0.5 to 100 ng. Epi-cortisol (500 ng) and D8-cortisone (250 ng) were used as internal standard in all samples and standards. Standard curves were generated using the ratio of analyte peak area to internal standard peak area. Interference between D4- and D3-cortisol in the analysis of samples was corrected for when quantifying peak areas. Standard curves were used to quantify analyte concentrations.

An enrichment curve of increasing D4-cortisol against a background of steady concentration of D3-cortisol showed lower than anticipated ratios of D4-cortisol: D3-cortisol peak area implicating unequal ionization of these analytes when co-existing in samples. Interference of one analyte to another was calculating in samples of

known concentrations of each (D4-cortisol and D3-cortisol) allowing tracer to tracee ratio in plasma samples to be adjusted accordingly.

5.5.3.2 Tracer kinetics

Rate of appearance of cortisol and cortisone as a measure of reductase and dehydrogenase activity by 11βHSD1 were measured as per Equation 7 and 8 below. Rate of appearance of D3-cortisol were calculated as described previously (Section 3.6.2).

Equation 7: Ra cortisol

$$\text{Ra cortisol (nmoll/min)} = \frac{\text{rate D4-cortisol infusion}}{\text{(D4-cortisol/cortisol)}}$$

Equation 8: Ra cortisone

$$\text{Ra cortisone (nmol/min)} = \frac{\text{rate of D2-cortisone infusion}}{\text{(D2-cortisone/cortisone)}}$$

Clearance of D4-cortisol was calculated by dividing the rate of infusion by its steady state concentration (L/min).

The rate of appearance of cortisol, D3-cortisol and cortisone across tissues was calculated using arteriovenous differences in tracer: tracee ratio whilst factoring in blood flow rate through the tissue as outlined below.

Equation 9: Cortisol release across tissue

Ra cortisol across tissue (nmol/l00 g tissue/min) =

$$BF \times [cortisol]_{artery} \times \frac{D4\text{-}cortisol/cortisol_{artery} - (BF \times [cortisol]_{artery})}{D4\text{-}cortisol/\ cortisol_{vein}}$$

Equation 10: D3-cortisol release across tissue

Ra D3-cortisol across tissue (nmol/l00 g tissue/min) =

$$BF \times [D3\text{-}cortisol]_{artery} \times \frac{D4\text{-}cortisol/D3\text{-}cortisol_{artery} - (BF \times [D3\text{-}cortisol]_{artery})}{D4\text{-}cortisol/\ D3\text{-}cortisol_{vein}}$$

Equation 11: Cortisone release across tissue

Ra cortisone across tissue (nmol/l00 g tissue/min) =

$$BF \times [cortisone]_{artery} \times \frac{D2\text{-}cortisone/\ cortisone_{artery} - (BF \times [cortisone]_{artery})}{D2\text{-}cortisone/\ cortisone_{vein}}$$

Where BF: blood flow (mL/min/100 g tissue) and [ ] denotes concentration.

5.5.4 Statistics

Data was analysed using SPSS statistics 23 (IBM). Steady state data was assessed between time+ 120 to 180 minutes. Difference between groups and phases was determined using a student's t-test. The influence of hyperinsulinaemia on glucocorticoid metabolism in whole body and muscle was determined using repeated measures analysis of variance (ANOVA) across the period +180 to +300 minutes.

Due to failure in plasma sampling from the intra-adipose cannula in the abdominal wall data for some individuals was incomplete. Individual missing data points were filled by taking the mean of the two surrounding values. Where few data points were available, missing data was left incomplete. For analysis of data from adipose tissue repeated measures ANOVA for the placebo/ insulin phase were limited to time + 270-300 minutes to maximize data for analysis.

All data are reported as mean ± SEM unless otherwise stated.

5.5.5 Results

5.5.5.1 Participant characteristics

One participant in the obese group was found to have developed a high fasting glucose level at his first visit. Data from this visit was excluded and his participation in the study was terminated. Ten lean (BMI 23.77 ± 0.38 kg/m^2) and ten obese (BMI 32.92 kg/m^2 ± 0.86 ($p<0.001$)) healthy male volunteers attended two study visits. Participants were matched for age (lean participants 50 ± 3.28 years; obese participants 50.5 ± 3.28 years). Obese participants had a significantly higher percentage body fat mass (obese 29.88 ± 1.41% vs lean 20.54 ± 1.94%; $p = 0.001$). Obese individuals had lower baseline plasma cortisol (lean 222.6 ± 47.2 vs obese 187.6 ± 41.3 nmol/L; $p=0.58$) and cortisone (lean 45.7 ± 6.7 versus obese individuals 39.4 ± 8.2 nmol/L; $p=0.56$).

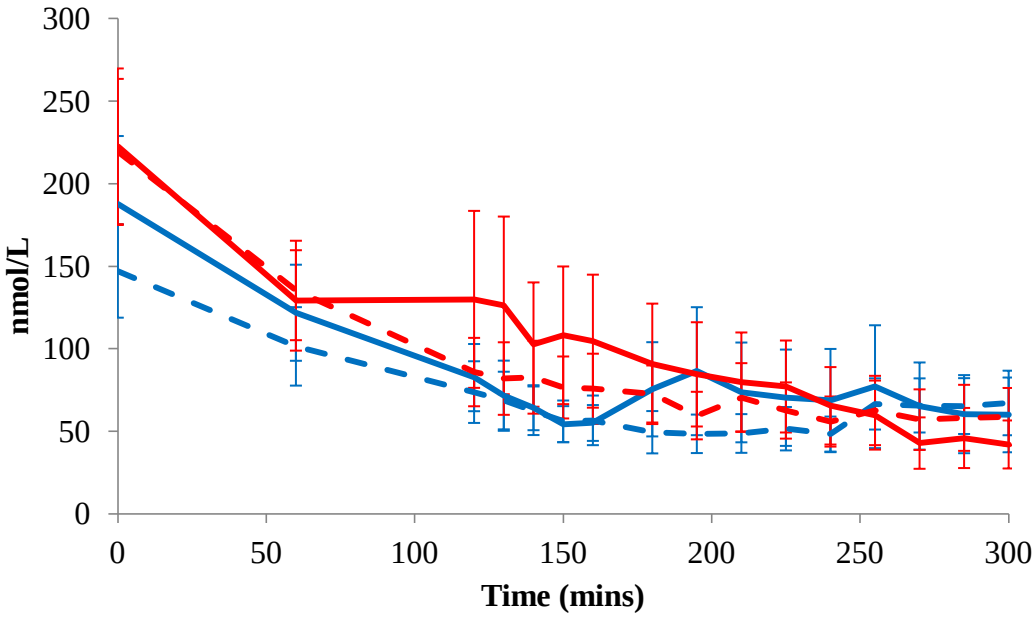
5.5.5.2 Steady state glucocorticoid kinetics before insulin/placebo infusion

5.5.5.2.1 Whole body glucocorticoid metabolism in steady state

During infusions, arterialised plasma cortisol and cortisone concentrations were similar between groups (lean vs obese individuals) and between visits (placebo vs hyperinsulinaemia) (Figure 5.5).

Arterialised D4-cortisol concentrations and enrichment with D3-cortisol were in steady state by 120 minutes. D2-cortisone and enrichment with cortisone had achieved steady state by the same time. The ratio of D4-cortisol: D3-cortisol (tracer: tracee ratio) appeared to be lower in arterialised blood in obese than lean individuals (Figure 5.6). This may be the result of a larger volume of distribution of tracer in obese individuals although this was not similarly reflected with the tracer D2-cortisone or with the ratio of D4-cortisol to cortisol (Figure 5.6).

(a) Cortisol



(b) Cortisone

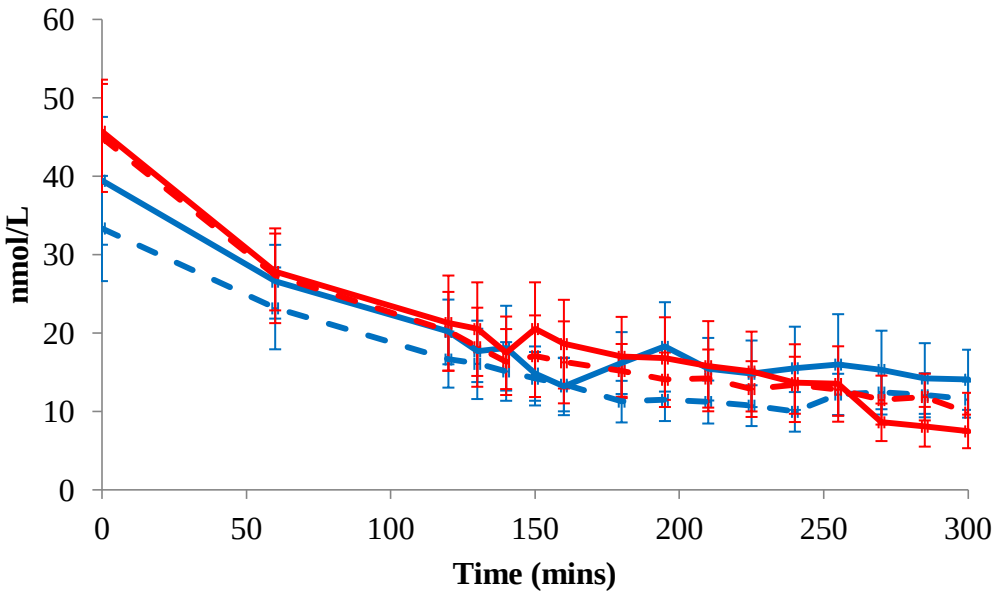


Figure 5.5 Concentration of cortisol and cortisone in arterialised blood

Where (a) cortisol; (b) cortisone. Blue solid line: obese placebo; blue broken line: obese insulin; red solid line: lean placebo; red broken line: lean insulin

(a) D4-cortisol: cortisol (ratio)

(b) D4-cortisol: D3-cortisol (ratio)

(c) D2-cortisone: cortisone (ratio)

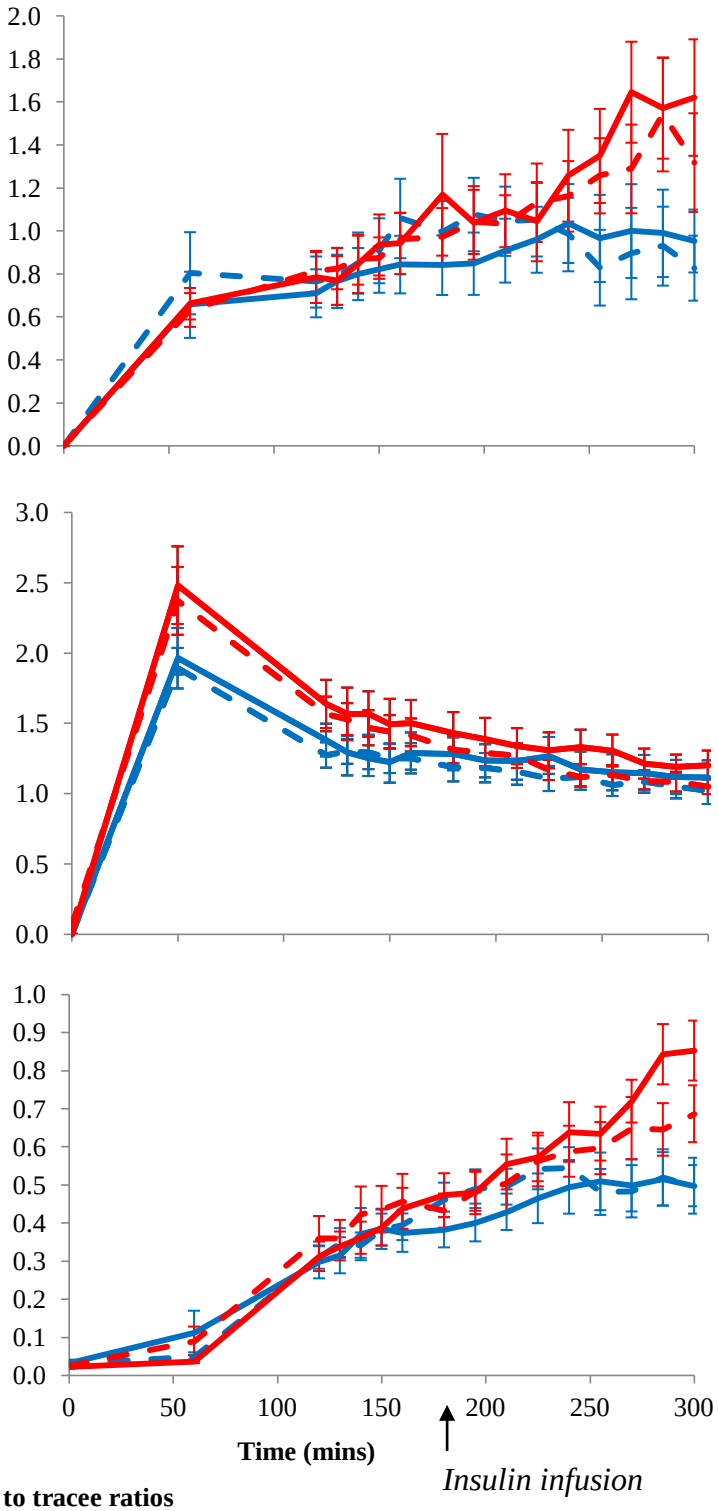


Figure 5.6 Arterialised tracer to tracee ratios

Arterialised tracer to tracee ratios for (a) D4-cortisol/ cortisol (b) D4-cortisol/ D3-cortisol and (c) D2-cortisone/ cortisone. Blue solid line: obese placebo; blue broken line: obese insulin; red solid line: lean placebo; red broken line: Lean insulin. Mean data ± SEM

Across whole body, rates of appearance of cortisol, D3-cortisol and cortisone did not differ significantly within each group between the two visits at steady state (pre-insulin vs pre-placebo infusion). No significant difference was observed in generation of cortisol, D3-cortisol or cortisone across whole body between lean and obese individuals (Table 5.1).

The clearance of D4-cortisol did not differ between groups (steady state: lean 0.47 ± 0.07 vs obese 0.54 ± 0.06 L/min; p = 0.49).

	Ra cortisol (nmol/min)		*Ra D3-cortisol (nmol/min)*		*Ra cortisone (nmol/min)*	
	Pre-placebo	***Pre-insulin***	***Pre-placebo***	***Pre-Insulin***	***Pre-placebo***	***Pre-Insulin***
Lean	58.20 ± 19.51	42.81 ± 6.95	22.66 ± 2.17	22.98 ± 1.81	15.34 ± 3.91	13.04 ± 2.07
Obese	55.79 ± 12.27	45.62 ± 8.42	26.17 ± 2.15	26.62 ± 1.94	15.82 ± 2.67	13.40 ± 1.65

Table 5.1 Steady state rates of appearance in whole body

Rates of appearance of cortisol, D3-cortisol and cortisone in whole body (nmol/min). Data are mean ± SEM. No difference was observed between the groups using t-test

5.5.5.2.2 Adipose tissue and skeletal muscle glucocorticoid metabolism in steady state

Blood flow through adipose tissue was significantly greater in obese compared with lean individuals during placebo phase (Figure 5.8) ($p<0.05$). Blood flow rate was similar through skeletal muscle in lean and obese individuals (Figure 5.9).

Arteriovenous differences in cortisol, cortisone and tracer: tracee ratios are shown in Figure 5.11 and Figure 5.12.

The resulting kinetic calculations for steady state are shown in Table 5.2. Across adipose tissue, the rate of appearance of cortisol, D3-cortisol and cortisone was different from zero in obese individuals only. Across skeletal muscle the rate of appearance of cortisol and cortisone was different from zero in lean individuals only.

At steady state, no significant difference was observed between the rate of appearance of cortisol, D3-cortisol or cortisone between phases (pre-insulin and pre-placebo) in skeletal muscle or adipose tissue. Comparison between groups at steady state was therefore performed using data from the placebo phase using student's t-test. Lean individuals displayed a significantly greater generation of cortisol in skeletal muscle than obese individuals (Ra cortisol; lean 20.44 ± 9.43 vs obese -13.51 ± 12.36 pmol/100 g tissue/min; $p<0.05$).

		Ra cortisol (pmol/100 g tissue/ min)		*Ra D3-cortisol (pmol/100 g tissue/ min)*		*Ra cortisone (pmol/100 g tissue/ min)*	
		Placebo	***Insulin***	***Placebo***	***Insulin***	***Placebo***	***Insulin***
Skeletal muscle	**Lean**	20.44 ± 9.43	18.62 ± 7.10†	4.87 ± 2.76	-9.57 ± 12.20	7.01 ± 1.61†	9.75 ± 1.83†
	Obese	-13.51 ± 12.36*	5.06 ± 3.62	13.11 ± 8.85	0.27 ± 3.11	6.83 ± 5.22	21.42 ± 12.34
Adipose tissue	**Lean**	39.93 ± 18.14	47.05 ± 27.15	2.89 ± 3.17	1.72 ± 1.23	3.41 ± 2.62	16.91 ± 13.24
	Obese	23.19 ± 8.54†	31.03 ± 14.14	10.25 ± 4.90	9.55 ± 3.20†	2.93 ± 0.57†	3.66 ± 1.46†

Table 5.2 Rates of appearance in skeletal muscle and adipose tissue

Rates of appearance of cortisol, D3-cortisol and cortisone in skeletal muscle and adipose tissue (nmol/min). Data are mean ± SEM.
* $p < 0.05$ for lean vs obese by independent sample t-test
† $p < 0.05$ compared with zero within each group in each phase

5.5.5.3 Effect of hyperinsulinaemia on glucocorticoid metabolism

With hyperinsulinaemia, mean glucose concentrations were maintained at physiological concentration (time t+ 180- 300 mins; lean placebo 5.32 ± 0.17 vs lean insulin 4.90 ± 0.13 vs obese placebo 5.25 ± 0.14 vs obese insulin 4.92 ± 0.26 mmol/L). Across time blood glucose concentrations were significantly higher early and lower later during insulin infusion compared with placebo in both lean and obese individuals (Figure 5.7).

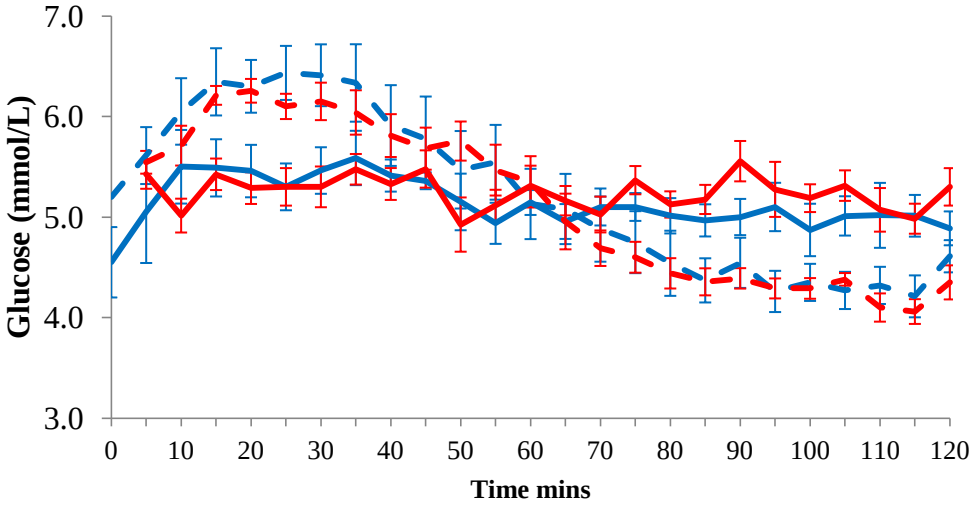


Figure 5.7 Glucose excursion

Glucose concentrations (mmol/L). **Blue solid line: o**bese placebo; **Blue broken line**: obese insulin; **Red solid line**: lean placebo; **Red broken line**; lean insulin. Data are mean ± SEM

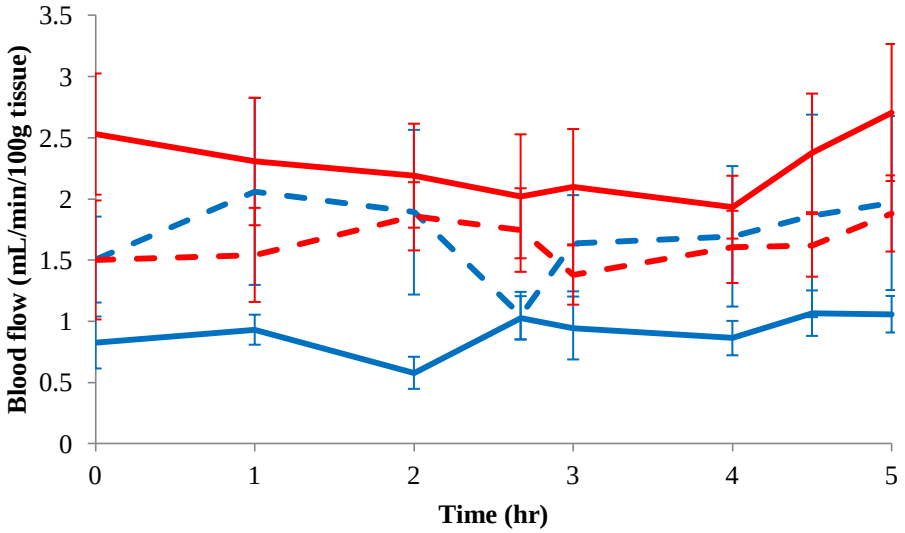


Figure 5.8 Blood flow adipose tissue

Blood flow as measured using Xenon^{133} washout. **Blue solid line: o**bese placebo; **Blue broken line**: obese insulin; **Red solid line**: lean placebo; **Red broken line**; lean insulin. Data are mean ± SEM. Lean vs obese (placebo phase) using student's t-test ($p<0.05$). Using two way repeated measured ANOVA there no significant effect of hyperinsulinaemia on blood flow rate ($p= 0.66$)

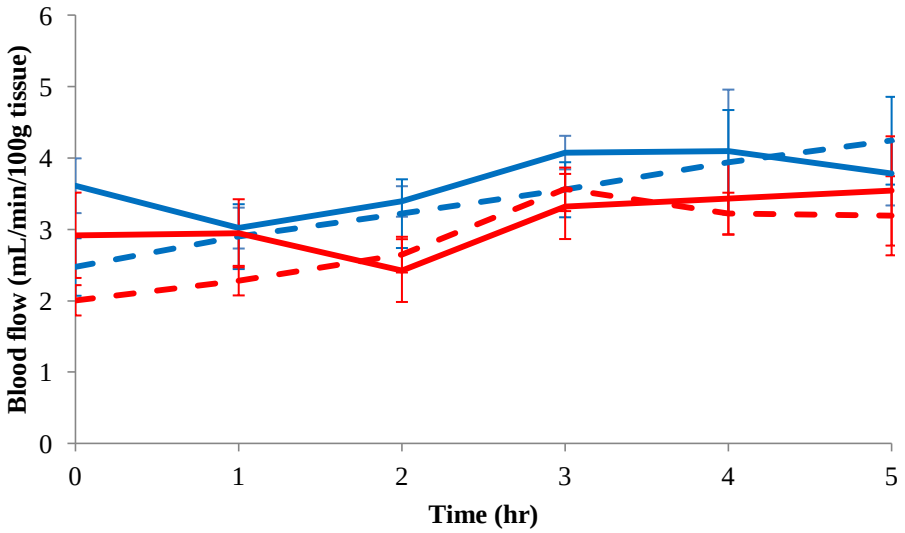


Figure 5.9 Blood flow in skeletal muscle

Blood flow as measured using venous occlusion plethsmography. **Blue solid line: o**bese placebo; **Blue broken line**: obese insulin; **Red solid line**: lean placebo; **Red broken line**; lean insulin. Data are mean ± SEM. Two way repeated measures ANOVA; overall p = 0.04; interaction with: subjects p = 0.99; obese vs lean p = 0.26; placebo vs insulin p = 0.47; lean vs obese vs placebo vs insulin = 0.71

5.5.5.3.1 Whole body glucocorticoid kinetics during insulin infusion

D4-cortisol clearance was not significantly altered by hyperinsulinaemia (t+ 180 – 300 mins: lean placebo 0.47 ± 0.07 vs lean insulin 0.45 ± 0.06 L/min: p= 0.27; obese placebo 0.54 ± 0.06 vs obese insulin 0.56 ± 0.07 L/min: p=0.62).

A two way repeated measures ANOVA was conducted to examine the effect of group and insulin treatment on the rate of appearance of cortisol, D3-cortisol and cortisone using absolute rates of appearance which are described in the text and in Table 5.3 but for clarity data are presented in the graphs as 'placebo-corrected' *i.e.*

by subtracting data during the placebo phase from that in the insulin phase for each subject (Figure 5.10).

Across whole body the rate of appearance of D3-cortisol and cortisone increased somewhat in lean individuals during the hyperinsulinaemic euglycaemic clamp although there was no significant difference between lean and obese individuals (Table 5.3 and Figure 5.10).

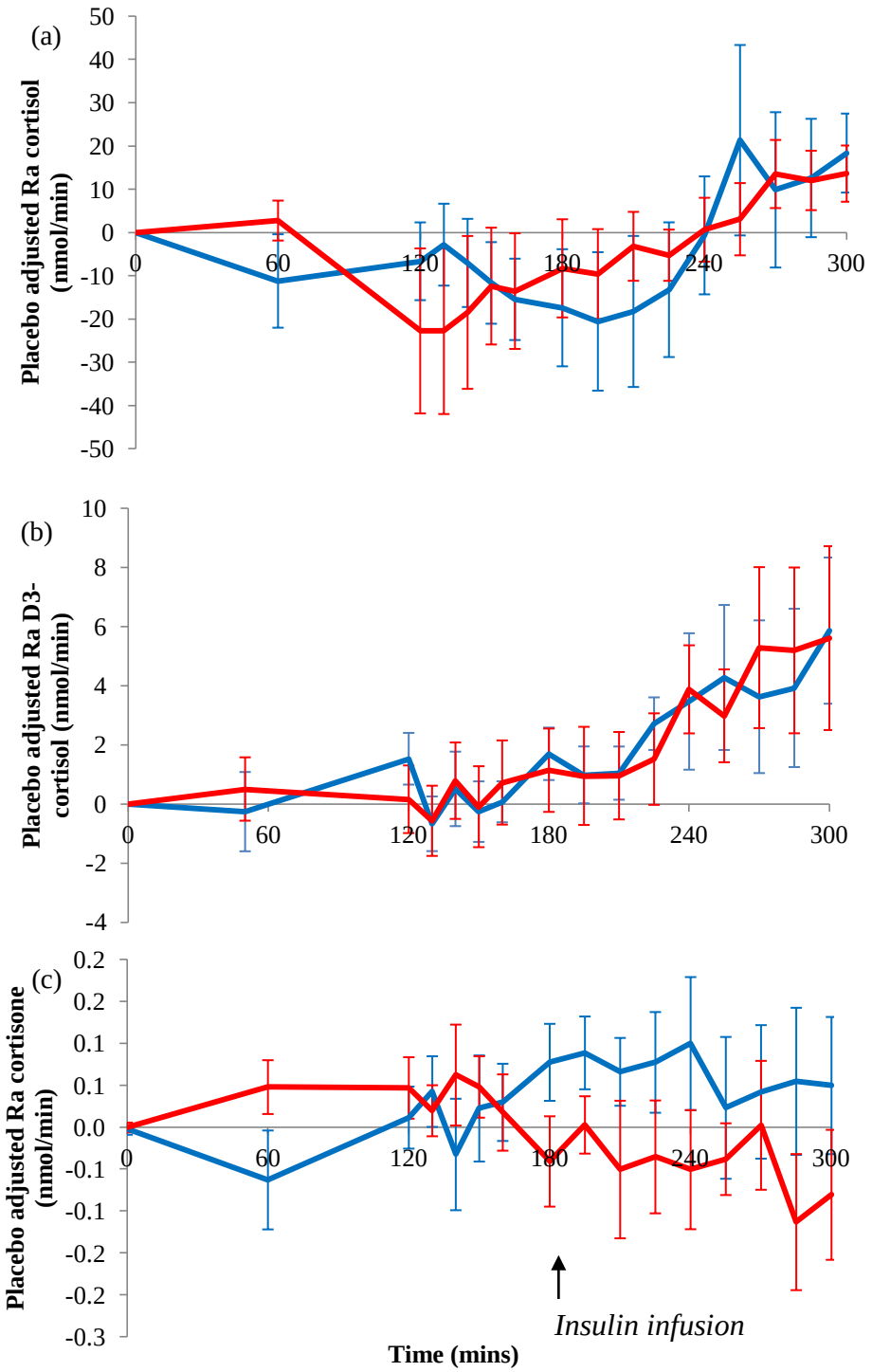


Figure 5.10 Placebo-corrected whole body rates of appearance of cortisol, D3-cortisol and cortisone during insulin infusion from t + 180 mins

Placebo adjusted rates of appearance of (a) cortisol (b) D3-cortisol and (c) cortisone in whole body pre- and following hyperinsulinaemia commenced at t = 180 mins.
Red line: lean individuals; **Blue line**: obese individuals. Data are mean ± SEM

	Ra cortisol (nmol/min)		Ra D3-cortisol (nmol/min)		Ra cortisone (nmol/min)	
	Placebo	Insulin	Placebo	Insulin	Placebo	Insulin
Lean	25.81 ± 5.14	35.65 ± 10.24	27.97 ± 2.72	30.51 ± 2.17*****	5.98 ± 0.64	7.99 ± 1.31*****
Obese	46.43 ± 11.22	54.23 ± 13.44	30.60 ± 3.21	31.92 ± 2.59	11.10 ± 2.5	11.12 ± 1.98

Table 5.3 Mean rates of appearance (Ra) of cortisol, D3-cortisol and cortisone in whole body during saline placebo infusion and with hyperinsulinaemia

Ra D3-cortisol: $p<0.001$; lean vs obese $p=0.68$; placebo vs insulin $p=0.026$
Ra cortisone: $p<0.001$: lean vs obese $p=0.47$; placebo vs insulin $p=0.06$

5.5.5.3.2 Adipose and skeletal muscle glucocorticoid kinetics during insulin infusion

Cortisol and cortisone concentrations were similar between arterialised and venous samples across adipose tissue and skeletal muscle in both lean and obese individuals (Figure 5.11 and Figure 5.12).

Statistical testing was restricted to rates of appearance after correction for blood flow for time+ 180-300 minutes for skeletal muscle and time+ 270-300 minutes for adipose tissue due to difficulties with sampling with the later. Data are presented for clarity as 'placebo corrected' in Figure 5.13 and mean rates of appearance in Table 5.4.

Across adipose tissue obese individuals tended to have increased rate of appearance of cortisol compared with lean individuals (Table 5.4). No significant difference was observed across this tissue for the rate of appearance of D3-cortisol or cortisone with hyperinsulinamia in either group. Additionally, no significant difference was observed for the rate of appearance of cortisol, D3-cortisol or cortisone across skeletal muscle in the lean or obese group.

As shown by the placebo corrected rates of appearance (Figure 5.13) results were very variable between individuals across time.

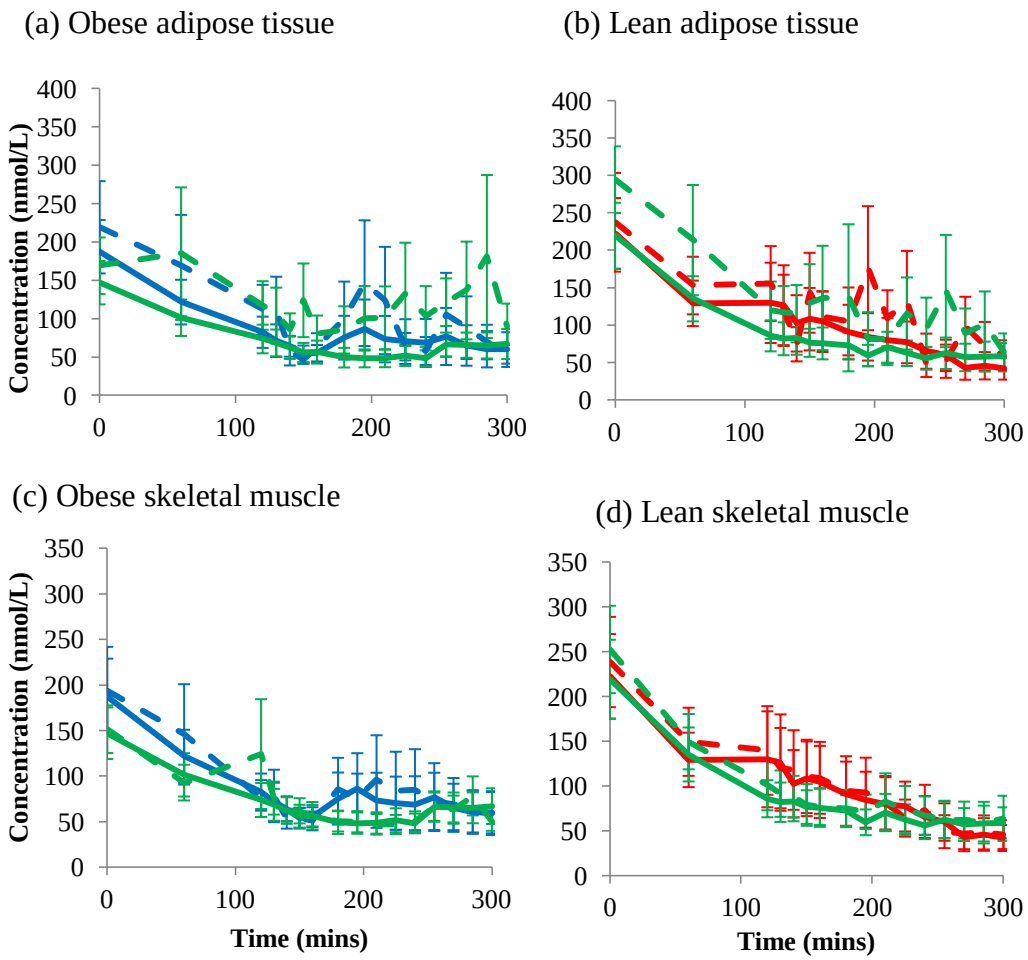


Figure 5.11 Arterio-venous differences in cortisol across adipose tissue and skeletal muscle during placebo saline infusion and hyperinsulinaemic euglycaemic clamp

Arterialised and venous concentrations (nmol/L) of cortisol across adipose tissue in (a) obese individuals and (b) lean individuals and across skeletal muscle in (c) obese individuals and (d) lean individuals.
Placebo phase - **Blue solid line**: arterial concentration; **Blue broken line**: venous concentration in obese individuals. **Red solid line**: arterial concentration; red broken line: venous concentration in lean individuals.
Hyperinsulinaemic phase – **Green solid line**: arterial concentration; **Green broken line**; venous concentration.

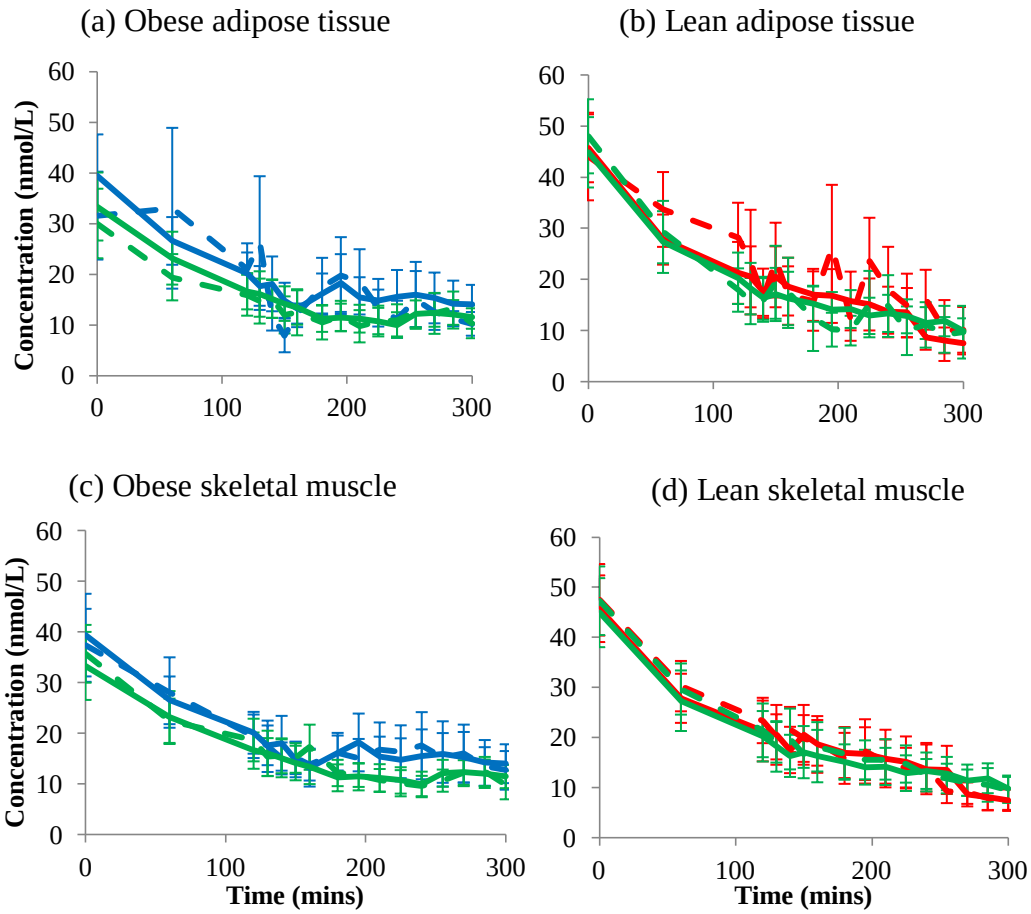


Figure 5.12 Arterio-venous differences in cortisone across adipose tissue and skeletal muscle during placebo saline infusion and hyperinsulinaemic euglycaemic clamp

Arterialised and venous concentrations (nmol/L) of cortisone across adipose tissue in (a) obese individuals and (b) lean individuals and across skeletal muscle in (c) obese individuals and (d) lean individuals
Placebo phase - **Blue solid line**: arterial concentration; **Blue broken line**: venous concentration in obese individuals. **Red solid line**: arterial concentration; **Red broken line**: venous concentration in lean individuals
Hyperinsulinaemic phase – **Green solid line**: arterial concentration; **Green broken line** venous concentration

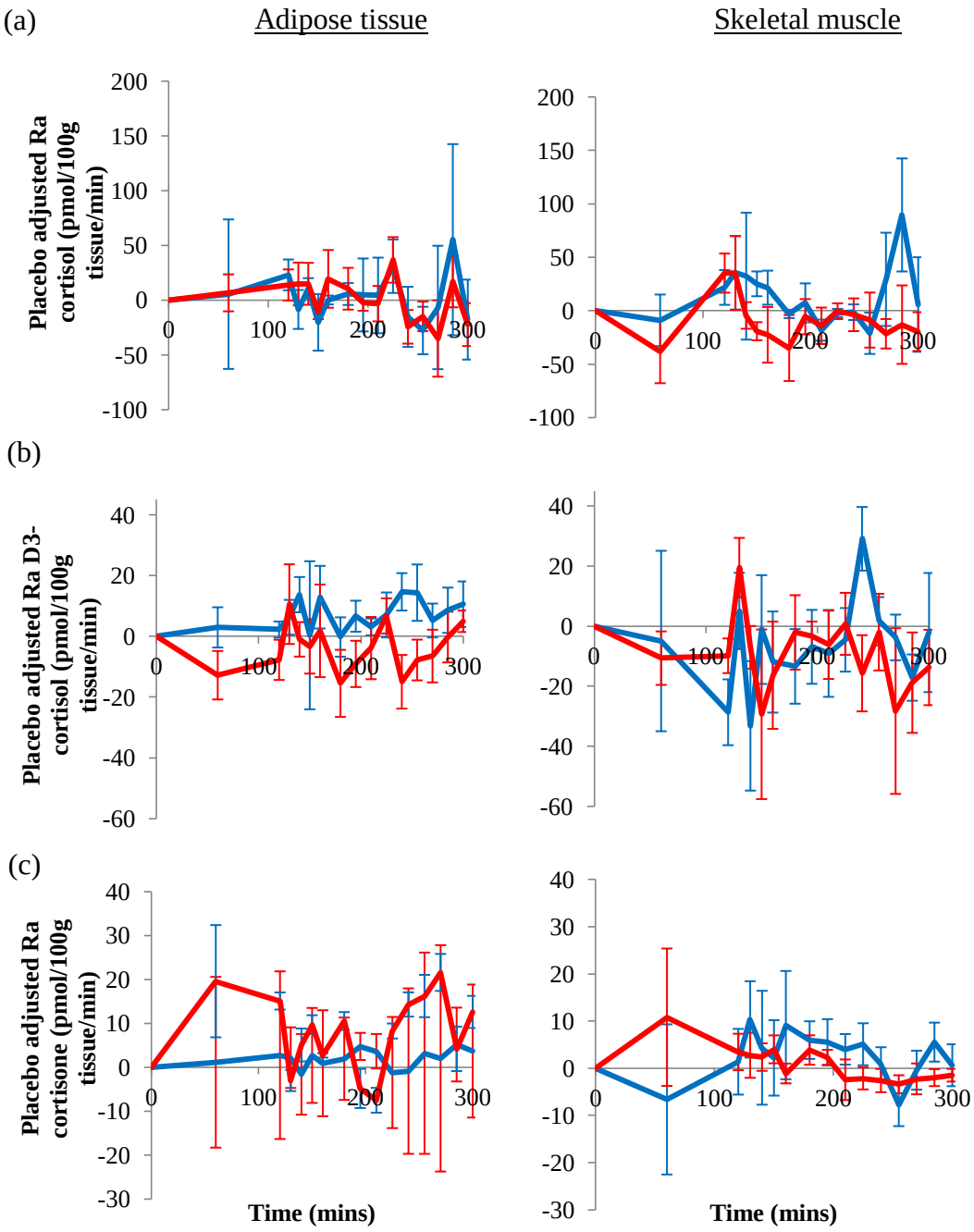


Figure 5.13 Placebo-corrected rates of appearance of cortisol, D3-cortisol and cortisone in adipose tissue and skeletal muscle during insulin infusion from t + 180 mins

Placebo adjusted rates of appearance of (a) cortisol (b) D3-cortisol and (c) cortisone in adipose tissue (right) and skeletal muscle (left) pre- and following hyperinsulinaemia commenced at t = 180 mins
Red line: lean individuals ; **Blue line**: obese individuals. Data are mean ± SEM

		Ra cortisol (nmol/min)		Ra D3-cortisol (nmol/min)		Ra cortisone (nmol/min)	
		Placebo	Insulin	Placebo	Insulin	Placebo	Insulin
Skeletal muscle	Lean	19.66 ± 11.94	6.80 ± 2.36	8.14 ± 3.22	-1.07 ± 7.07	9.75 ± 1.83	3.62 ± 1.26
	Obese	1.24 ± 4.28	11.98 ± 7.91	9.24 ± 3.5	5.31 ± 2.55	11.15 ± 3.69	5.63 ± 1.78
Adipose tissue	Lean	67.78 ± 93.52	44.35 ± 90.89	5.27 ± 3.58	3.53 ± 3.72	5.60 ± 1.39	18.29 ± 17.52
	Obese	48.46 ± 31.76	56.20 ± 31.85*	4.9 ± 2.73	18.15 ± 8.53	1.82 ± 2.3	3.73 ± 1.57

Table 5.4 Mean rates of appearance (Ra) of cortisol, D3-cortisol and cortisone across adipose tissue during saline placebo infusion and with hyperinsulinaemia

Ra cortisol: $p<0.01$; lean vs obese $p=0.62$; placebo vs insulin $p=0.11$

5.5.5.4 Tissue specific reductase: dehydrogenase activity

The ratio of rate of appearance of D3-cortisol (Ra D3-cortisol) to rate of appearance of cortisone (Ra cortisone) provides a comparative measure of rate of reductase to dehydrogenase activity by 11βHSD1 activity across tissue. Across skeletal muscle during steady state (Table 5.1), the ratio of Ra D3-cortisol to Ra cortisone was predominantly dehydrogenase (ratio = 0.2) whilst in obese individuals it was predominantly reductase (ratio = 1.41). Across adipose tissue, lean individuals again

showed predominance of dehydrogenase activity (ratio = 0.26) with predominantly reducatsae activity in obese individuals (ratio = 3.26).

5.5.6 Discussion

Using deuterated tracers for the quantification of 11β-reductase and 11β-dehydrogenase activity, we have shown similar 11βHSD1 activity in whole body between lean and obese individuals. This corroborates with previously published data (Dube et al. 2014; Sandeep et al. 2005). Across tissues, obese individuals had quantifiable 11β- reductase and 11β-dehydrogenase activity that was different from zero in subcutaneous adipose tissue but not in skeletal muscle. In lean individuals the appearance of cortisol and cortisone was significantly different from zero in skeletal muscle only. Unlike the generation of D3-cortisol, the appearance of cortisol may reflect increased endogenous adrenal production.rather than 11βHSD1 activity. With acute perturbation of the enzyme with hyperinsulinaemia, whole body 11β-reductase and 11β-dehydeogenase activity was increased although there was no significant difference between the two groups. This may reflect increases insulin sensitivity in lean individuals exemplifying the role of insulin in increasing 11βHSD1 activity. Across tissue, there was a trend for hyperinsulinaemia to increase the production of cortisol in obese but not lean individuals. This may reflect adrenal synthesis rather than 11βHSD1 activity. It is additionally recognised that due to slow accumulation of D4-cortisol into tissue such as subcutaneous adipose and hence slow generation of D3-cortisone, 11β reductase activity may be reflected through the generation of cortisol rather than through the anticipated D3-cortisol production (Hughes, et al. 2010).

The measurement of glucocorticoid kinetics across tissue may be influenced by blood flow across that tissue. Obese individuals displayed significantly lower blood flow rates during the placebo but not hyperinsulinaemic phase. Blood flow rates following an overnight fast have been reported to be around 3 mL/100g tissue/min in adipose tissue and 1.5 mL/100g tissue/min in skeletal muscle (Elia and Kurpad 1993). Our study found slightly lower blood flow rates through adipose tissue yet slightly higher rates through skeletal muscle. It is recognised that fasting (Hagstrom-Toft, et al. 1997; Klein, et al. 2000) and mental stress (Linde, et al. 1989) can increase whilst obesity (Jansson, et al. 1998) and insulin resistance (Karpe, et al. 2002) can lower blood flow rate in tissue.

Previous studies have shown upregulated 11β-reductase activity in adipose tissue with obesity (Rask et al. 2001; Stimson et al. 2009). Although we did not observe any significant difference in 11βHSD1 activity between lean and obese individuals, we have shown quantifiable reductase and dehydrogenase activity in this tissue in obese but not lean individuals. The significance of adipose tissue in cortisol generation is additionally highlighted by comparing the rate of reductase to dehydrogenase activity between lean and obese individuals with predominantly dehydrogenase activity in the former and reductase activity in the later.

A recent study using a novel triple tracer technique also observed no significant difference in the rate of appearance of cortisol across subcutaneous adipose tissue in obese compared with lean individuals (Dube et al. 2015a). This study used microdialysis catheters for sampling across tissue. The smaller sample volume generated by this technique may impact on results especially if tracer enrichment

within the tissue is low or if alterations in tracer to trace ratio are of a small magnitude.

Across skeletal muscle we observed production of cortisol, D3-cortisol and cortisone in lean individuals only. Few studies have investigated glucocoticoid kinetics across skeletal muscle *in vivo*. A study performed by Basu et al. showed in a cohort of obese individuals D3-cortsol production did not differ from zero both before or during an insulin infusion (Basu et al. 2004). A study performed within our department showed quantifiable rates of production cortisol, D3-cortisol and cortisone across forearm skeletal muscle in lean/overweight men (Hughes et al. 2012).

With hyperinsulinaemia only adipose tissue displayed alteration in the rate of appearance of glucocorticoids with a trend for increased cortisol generation in obese individuals. The appearance of cortisol across this tissue may reflect increased adrenal synthesis but could still conceivably be due to increased 11βHSD1 activity which is masked by the slow accumulation of D4-cortisol into the tissue as discussed above. Upregulation of adipose 11βHSD1 activity has been observed in a previous *in vivo* study following insulin infusion (Wake et al. 2006). *In vitro* data has shown that insulin has no effect on availability of coenzyme H6PDH but does extend the half life of 11βHSD1 mRNA two fold (Balachandran et al. 2008). By driving glucose into cells, insulin can increase substrate for H6PDH generating more NADPH and hence driving 11βHSD1more in a reductase than deydrogenase activity. This relies on sensitivity of the cell to the circulating insulin. Glucose uptake into tissue such as muscle is additionally stimulated by movement. Although the participants were

restricted in their movement due to the number of attachments for blood sampling, intravenous infusions and blood flow measurement this may falsely elevate the measured 11βHSD1 activity.

With 11βHSD1 activity up to 2.5 fold higher per gram of adipose tissue in obese compared with lean controls (Rask et al. 2001; Rask et al. 2002) the difference in response to insulin between groups seen in this study may reflect the variation in target organ mass.

From our *in vitro* data (Section 5.4) and previous *in vivo* work (Hughes et al. 2012), 11βHSD1 has been shown to catalyse both the reduction of cortisone to cortisol and dehydrogenation of cortisol to cortisone. The generation of cortisone across adipose tissue and skeletal muscle in this study is thought to be the result of dehydrogenation by 11βHSD1. It is, however, recognised that our current D2-cortisone tracer cannot distinguish between the activity of 11βHSD1or 2. The localisation of 11βHSD2 in adipose tissue (Bujalska et al. 1999; Engeli et al. 2004; Lee et al. 2008) and skeletal muscle (Abdallah et al. 2005; Hassan-Smith et al. 2015; Jang et al. 2007; Whorwood et al. 2002) has been variable been studies. *Ex vivo* biopsies from this cohort would allow clarification of the isoenzyme responsible.

With confirmed bidirectional activity of 11βHSD1 *in vivo* adverse metabolic phenotypes may be represent dysregulation at a cellular level of disparate reactions by the enzyme rather than overall enzyme activity. With a 3 to 1 ratio of reductase to dehydrogenase activity in subcutaneous adipose tissue the importance of this tissue in the development of obesity and the metabolic syndrome is reinforced (Bujalska et al. 1999). In corroboration with our tissue kinetics, a similar study investigating

11βHSD1 across tissue in lean and obese individuals showed increased oxo-reductase activity in adipose tissue of obese individuals but with no difference across forearm skeletal muscle (Katz, et al. 1999).

5.6 Conclusion

In conclusion, in this chapter we have quantified bidirectional activity of 11βHSD1 *in vitro* and *in vivo* using deuterated tracers and shown that obese individuals display predominantly 11β-reductase activity across tissue. Reductase specific inhibitors may prove more efficacious for the treatment of obesity.

Chapter 6: Conclusions

From initial recognition of the similarities between Cushing's syndrome and the increasingly prevalent metabolic syndrome, to the development of 11βHSD1 inhibitors as a novel means for the treatment of T2DM, our understanding of cortisol regulation and dysregulation has increased greatly over the past twenty years. The lack of direct relationship between circulating cortisol and measures of obesity implicated more complex regulation which was has been proven through observations of tissue specific dysregulation in both obesity and T2DM. Differing regulation between tissues in these two conditions provides evidence of the adaptive ability of 11βHSD1. Unlike in murine models whole body suppression of enzyme activity in humans has shown poor efficacy for the treatment T2DM and associated metabolic features. This thesis aims to explore the potential reasons for this by investigating cortisol regulation by 11βHSD1 at a cellular level.

Inhibitors of 11βHSD1 have been developed for the treatment of T2DM with anticipated beneficial effects on cardiovascular risk factors including obesity. Few agents have reached phase 2 trials and their efficacy has been disappointing. If used commercially, such agents would likely be prescribed alongside the longstanding first line treatment, metformin. With the mechanism of action of metormin remaining uncertain, any role of metformin on 11βHSD1 activity may mask the full potential of these novel inhibitors.

The effect of metfomin on 11βHSD1 activity was investigated in obese men with and without T2DM in a randomised placebo controlled double blinded cross over trial.

Through the use of the deuterated tracer D4-cortisol, whole body 11βHSD1 activity was increased following 28 days of metformin treatment by 10 to 15% (Ra D3-cortisol placebo vs metformin respectively: OND; 11.2 ± 1.4 vs 12.9 ± 1.2 nmol/min $p<0.05$: ODM 14.21 ± 0.63 vs 15.78 ± 0.58 nmol/min $p<0.01$). This was independent of any glucose lowering effect by metformin as the additional control phase in the ODM with gliclazide treatment showed no significant effect on 11βHSD1 activity (Ra D3-cortisol OND: placebo 14.21 ± 0.63 vs metformin 15.78 ± 0.58 vs gliclazide 14.21 ± 0.54 nmol/min). Obese individuals with T2DM were additionally observed to have increased whole body 11βHSD1 activity in comparison to the OND group (Ra D3-cortisol OND 11.2 ± 1.4 vs 14.21 ± 0.63 nmol/L; $p<0.05$). Contrary to our hypothesis, metformin appears to increase whole body 11βHSD1 activity suggesting that any reduced efficacy of 11βHSD1 inhibtiros is not through the co-administration of metformin.

As a key site of splanchnic cortisol regeneration by 11βHSD1 (Andrew et al. 2005; Basu et al. 2009) and principle target of metformin, the liver was hypothesised as the likely site of any alteration in 11βHSD1 activity. Although this was not proven, it was suggested through the finding of a trend for upregulation of hepatic 11βHSD1 by metformin in the ODM group. This was however a secondary outcome which the study was underpowered to detect. It is proposed that through improvement in insulin sensitivity in the liver, metformin increases cellular glucose and, through the action of H6PDH, increases availability of co-substrate. Measurement of insulin resistance using HOMA-IR showed a non-statistically significant reduction with metformin treatment. Previous work has shown a lack of acute effect of an alternative insulin sensitizing agent, namely pioglitazone in down regulating 11βHSD1 mRNA and

activity in adipose tissue, at least acutely. More chronic treatment over 12 weeks did downregulate 11βHSD1 mRNA (Wake, et al. 2007). Direct measurement of enzyme activity across liver is hindered by feasibility of access to hepatic blood supply. The use of hepatocytes *in vitro* would allow measurement of 11βHSD1 activity in the presence or absence of metformin and identification of a potential causative pathway.

Due to its significant contribution to circulating cortisol concentrations, the most likely alternative causative tissue would be adipose. It would therefore be of interest to explore the direct effect of metformin on adipocyte 11βHSD1 either directly using arterio-venous sampling across the tissue or using *ex vivo* samples from subjects following administration of metformin.

Isotopic tracers have allowed whole body and tissue specific measurement of 11βHSD1 activity. Calculation of enzyme kinetics relies on plasma sampling and measurement of dilution of one isotope with another. Only unbound or 'free' glucocorticoids can pass across the cell membrane into the cytosol to be metabolised by 11βHSD1. Glucocorticoids are actively exported out of the cell back into the circulation with only around 5% remaining unbound within the circulating plasma pool. The remainder binds to carrier proteins such as CBG and albumin. Clinical studies to date have relied on measurement of total plasma glucocorticoids *i.e.* free plus bound for measurement of 11βHSD1 activity. This assumes equal enrichment in both the free and bound circulating pools with rapid turnover between the two. If this does not hold true then quantification of whole body 11βHSD1 activity to date may

have been underestimated and therefore have implications on the perceived contribution of tissues on whole body enzyme activity.

With no ‘gold standard’ method available an assay was developed for isolation of unbound cortisol. Plasma samples from a clinical study previously performed within the department investigating the effect of isocaloric high carbohydrate, high protein and placebo meal on 11βHSD1 activity were used to compare the enrichment of D4-cortisol by both cortisol and D3-cortisol between the free and total pools. At pre steady state and steady state infusion of D4-cortisol the rate of appearance of endogenous and D3-cortisol was reflected similarly in the free and total plasma pool. The effect of acute perturbation of this equilibrium was assessed using samples taken following administration of the isocaloric meal. Again the rate of appearance of both glucocorticoids was reflected similarly in the free and total pool. It is therefore evident that at a whole body level glucocorticoids turnover rapidly between the free and total pool. Through alterations in binding affinity this may not hold true in the presence of chronic inflammation such as with obesity or at sites of local infection and injury. Further investigation of the rate of turnover between the two pools across individual tissues and comparing lean, obese euglycaemic and obese individuals with T2DM may provide further insight into the patho-physiological basis underlying these conditions.

Until recently, 11βHSD1 has been regarded principally as a reductase enzyme *in vivo*. This has been disproven through the use of a deuterated cortisone tracer and identification of dehydrogenase activity in both adipose tissue and skeletal muscle

(Hughes et al. 2012). The contribution of this activity to overall whole body glucocorticoid metabolism and the effect of metabolic disturbance with obesity remained unknown. The ability for 11βHSD1 to act as a dehydrogenase as well as a reductase enzyme was confirmed *in vitro* using HEK 293-11βHSD1 cells. A random-order two-phase crossover single-blinded study was subsequently designed to investigate 11βHSD1 directionality in lean and obese healthy male volunteers.

During steady state tracer infusion, we observed no significant difference in whole body rate of appearance of cortisol, D3-cortisol or cortisone between lean and obese individuals. Across tissue, oxo-reductase activity was statistically different from zero across adipose tissue in obese individuals and across skeletal muscle in lean individuals. By taking the ratio of reductase to dehydrogenase activity across tissue it was evident that 11βHSD1 acts as a predominatly reductase enzyme in adipose tissue and skeletal muscle in obese individuals but as a predominantly dehydrogenase enzyme in these tissues in lean individuals. Enzyme activity was subsequently perturbed using a hyperinsulinaemic-euglycamic clamp. During this phase, lean individuals showed an increase in oxo-reductase activity by 11βHSD1 in whole body and across tissue obese individuals tended to have increased cortisol generation. This suggests that inhibitors specifically targeting reductase activity by 11βHSD1 may prove more efficacious for the treatment of obesity related disorders such as T2DM.

In this study we have confirmed bidirectional activity of 11βHSD1 *in vivo* and shown that in the setting of obesity 11βHSD1 in tissue acts as a predominantly reductase enzyme. With the finding of balanced reductase and dehydrogeanse activity in whole body with obesity, it is possible that this increase in reductase

activity is counterbalanced by net deyhdrogenase activity in alternative tissues such as liver. Further studies are needed to investigate this. With our findings in Chapter 3 of increased whole body reductase activity in individuals with T2DM supported by previous published data (Stimson et al. 2011), it would also be of interest to investigate whether 11βHSD1 activity is predominatly dehydrogenase in OND individuals yet reductase in ODM individuals.

To conclude, this thesis has detailed factors influencing cortisol regulation at an intracellular level and discussed the impact of this at whole body level in relation to the metabolic syndrome and T2DM.The development of novel pharmaceutical agents for the treatment of obesity and T2DM has been explored elucidating potential pitfalls and as such identifying areas for potential future development.

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